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14. ABSTRACT
We examined whether chronic exposure to combinations of two neurotoxicants (chlorpyrifos and permethrin) with pyridostigmine bromide (PB) could produce a delayed neuropathic pain condition in rats; and whether corresponding molecular changes would occur in nociceptive neurons coding for pain in skin, muscle or vasculature. Following a 60 day exposure to neurotoxicants/PB (NTPB), we observed molecular dysfunctions in membrane Kv (Kv7) and Nav (Nav1.8, Nav1.9) proteins that persisted 8 weeks after exposure had ended. Functional changes (spontaneous activity, action potential duration) were also documented. Most of the maladaptations were present in vascular nociceptors. The physical location of vascular nociceptive neurons renders them most exposed to concentrations of circulating neurotoxicants/PB as well as to any blood borne secondary influences (endocrine, immune) these agents might induce. As a result, vascular nociceptors could be the first nervous system component damaged by neurotoxicants/PB. An imbalance between Kv_7 and other Na^+ channel proteins (Nav1.9) could prove to be a basis for a chronic pain condition sourced from a vulnerable subset of vascular nociceptors. A resulting neurovascular reflex dysfunction could cause widespread pain and also contribute to the development of CNS symptoms that have been identified in Gulf War veterans.

15. SUBJECT TERMS
Gulf War Illness, Pain, Neurotoxicants, Insecticides, Pyridostigmine Bromide, nervous system

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Introduction

Chronic, widespread joint and muscle pain is a frequent and debilitating component of GWI syndrome (Gulf War Illness; Stimpson et al., 2006; Thomas et al., 2006). The events leading to the development of GWI pain are unknown, but it has been reported that the use of pyridostigmine bromide (PB) and a variety of insecticides/repellants co-varied with the development of this condition (Research Advisory Committee on Gulf War Veterans' Illnesses, 2008; Steele et al., 2011). In the SOW (statement of work), we outlined a series of behavioral and electrophysiological (molecular) studies to determine the dose combinations of pyridostigmine bromide (PB), permethrin and chlorpyrifos that produced a condition of persistent muscle pain in a rodents. These behavioral studies were supported by neurophysiological experiments to determine the molecular basis of the chronic pain and provide a specific direction for the exploration of treatments to ameliorate the condition. The hypotheses were guided, in part, by the known acute effects of permethrin on the structurally and functionally similar insect and mammalian protein, $Na_v1.8$ (TTX resistant VGSC; Choi and Soderlund, 2006), and the high degree of association of this protein with mammalian nociceptors. Behavioral studies assessed concurrent and delayed manifestation of muscle pressure pain that followed 30-60 days exposure to combinations of the neurotoxicants with PB. Molecular studies were conducted, 4-12 weeks after dosing, in specific sub-populations of nociceptors that innervated skin, muscle and vasculature.

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Synergisms that might occur between insecticides, repellants and nerve agent prophylactics used during the GW could modify or damage the machinery of protein expression in ways that ultimately result in chronic pain and other symptoms of GWI (Bradberry et al., 2005). The Research Advisory Committee on Gulf War Veterans' Illnesses (Binns et al., 2008) emphasized a number of agents with the potential to produce such synergisms. An animal model that

reproduces the pain symptoms of GW veterans would be a valuable asset to the discovery of a molecular defect in the pain system. This discovered molecular defect might be generalized to other neuronal populations in the CNS with wider implications for GWI syndromes. We used a rat model of muscle pain to provide a means to assess the influence of insecticides and PB on sensory processing.

In the EOY report below, the findings of year 1 are interwoven with the findings of year 2. To clarify the progress made in different periods, those studies designated as NTPB were all conducted in year 2.

Chronic Influences of Neurotoxicants and PB on Pain Behavior

Task 1: We will examine the development, time course and persistence of a myalgia in rats exposed to combinations of neurotoxicants pyridostigmine bromide, permethrin and chlorpyrifos.

Two neurotoxicant/PB dosing regimens were completed during the previous reporting period (NR1 and NR2). These were complemented and extended by two additional treatment protocols that were based on a single dosing regimen (NR3/NTPB). These specific dose regimens are presented in Table 1. In both NR1 and NR2, treatments continued for 30 days, after which a 4 week delay passed before animals were sacrificed for electrophysiological experiments. In NTPB, treatments continued for 60 days and molecular and cellular tests were made 8 and 12 weeks after treatment had ceased. Delays were instituted to ensure that any pain that developed from the synergism of neurotoxicants would persist, like GWI pain, after the exposure had ended (Kroenke et al., 1998).

Behavioral Studies. Young adult male rats were treated with permethrin and chlorpyrifos along with a standard military dose of pyridostigmine bromide (assuming 70 kg body weight). Treatments continued for 30 or 60 days. Pressure pain measures were taken using a computer regulated, hand held test device by which pressure was applied via transducer to the semitendinosus and biceps femoris muscles (right hind limb; PAM, Ugo Basile). To complement pressure pain testing, activity levels (movement distance, rest time) were recorded

automatically in an activity box (15 min test period; AccuScan). It was hypothesized that hind limb withdrawal threshold and movement distance indices would decrease in animals experiencing widespread pain while rest periods would increase. Behavior tests were assessed in both treated (permethrin, chlorpyrifos, PB) and vehicle (ETOH, corn oil, water) exposed animals over an identical time course. Behavior testing occurred in double blinded conditions.

Table I

	PB mg/kg, Oral	Permethrin mg/kg, Topical	Chlorpyrifos mg/kg, SC	Exposure days
NR1	13	1.3	60	30
NR2	13	2.6	120	30
NR3	13	2.6	120	60

The duration of dosing different as described in the text
NTPB refers to dosing at the NR3 level and is an equivalent term

When combinations of neurotoxicants and PB were administered in NR1 and NR2, transient pain appeared only in NR2 and NR3/NTPB, and was found to be associated with molecular maladaptations in a specific nociceptor sub-population (vascular nociceptors). The changes we observed did not persist past the dosing period. Nevertheless we did observe a number of molecular and cellular changes 4, 8 and 12 weeks after the dosing had ceased. The lack of concurrent behavioral and molecular changes could have indicated that: 1) the molecular shifts were pre-clinical, and that longer dosing periods or additional agents were required to observe simultaneous pain and molecular defects; 2) the molecular shifts were compensatory to the pain previously manifested but that had since subsided; 3) the muscle pain sensitivity test was not

appropriate for what might have been a vascular pain condition. Detailed behavioral findings from both reporting periods are shown below:

The 30 day treatment with NR1 did not produce changes in behavioral pain threshold measures (figure 1A). However, after a 30 day exposure to NR2, we did observe significant decreases in muscle pain threshold during the NR2 dosing period. The decreases in muscle pain subsided and actually reversed during the weeks that followed dosing of the animals. Other pain correlates, such as rest times, also shifted, consistent with a pain condition, during dosing with neurotoxicants, but eventually reversed polarity 4 weeks after treatments had terminated (figure 1B). In contrast, movement distance measures mainly increased relative to controls in all treatment conditions and periods. The increase in movement distance coupled to an increase in rest times indicated more rapid movement during periods of activity, as the overall time of observation was fixed at 15 minutes. Overall, the behavior data indicated, at best, that the higher doses of neurotoxicants and PB could produce a transient increase in the muscle pressure pain, but only during the 4 weeks of dose application.

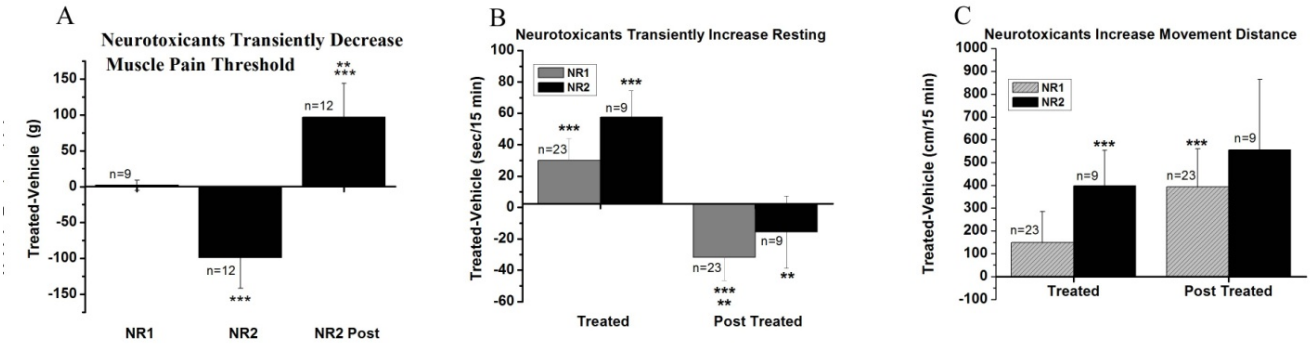

Figure 1. Neurotoxicants Transiently Decrease Pain Threshold and Increases Resting Time. A) Muscle pain threshold drops during NR2 treatment relative to controls, but reverts after treatment ceases. **B)** Neurotoxicant-induced rest time increases paralleled pain measures in NR2 (black bars). **C)** Rat movement distance increases with both NR treatments, suggesting faster movement in less time. The data are presented as difference scores between vehicle and dosed animals (double blinded tests). Activity measured by automated collection system in 15 minute observation periods. *** significantly different from vehicle dosed; ** significantly different from rats concurrently dosed with neurotoxicants.*

In an effort achieve a behavioral effect, we increased the exposure period to 60 days while keeping the dosages at the same level (NTPB). Sixty days was a duration that was consistent with deployment in the Persian Gulf War. During a 60 day exposure to NTPB, we observed increased resting behaviors that may have been associated with pain or malaise, but this only occurred during periods that coincided with neurotoxicant dosing (coincident data not shown). At the 8 week and 12 week delay periods, there was no indication of a change in hindlimb withdrawal or ambulatory behaviors of the treated rats (figure 2). Accordingly, there was little indication that this behavioral model and exposure regimen could detect or quantify a widespread delayed onset pain of the sort reported by returning veterans (Kroenke et al., 1998; Stimpson et al., 2006; Thomas et al., 2006). While this did not rule out that some persistent pain, unmeasured with these methods, might nonetheless be present, the failure of the behavior model to measure any pain in the weeks following NTPB exposure prevents any strong association of the molecular changes with a functional behavioral influence of these agents. Other behavioral measures and/or other treatment regimens may be required.

The failure to detect a persistent pain did it rule out that the animals might experience mild pain that we were unable to detect or pain from tissue sources that were not assessed by the muscle pressure apparatus (e.g., joint or vascular pain). Accordingly, as described in the SOW, we examined whether changes Na_v or K_v7 proteins were altered in any way by neurotoxicant/PB exposure (NTPB). Studies were conducted on the same animals that were behaviorally tested.

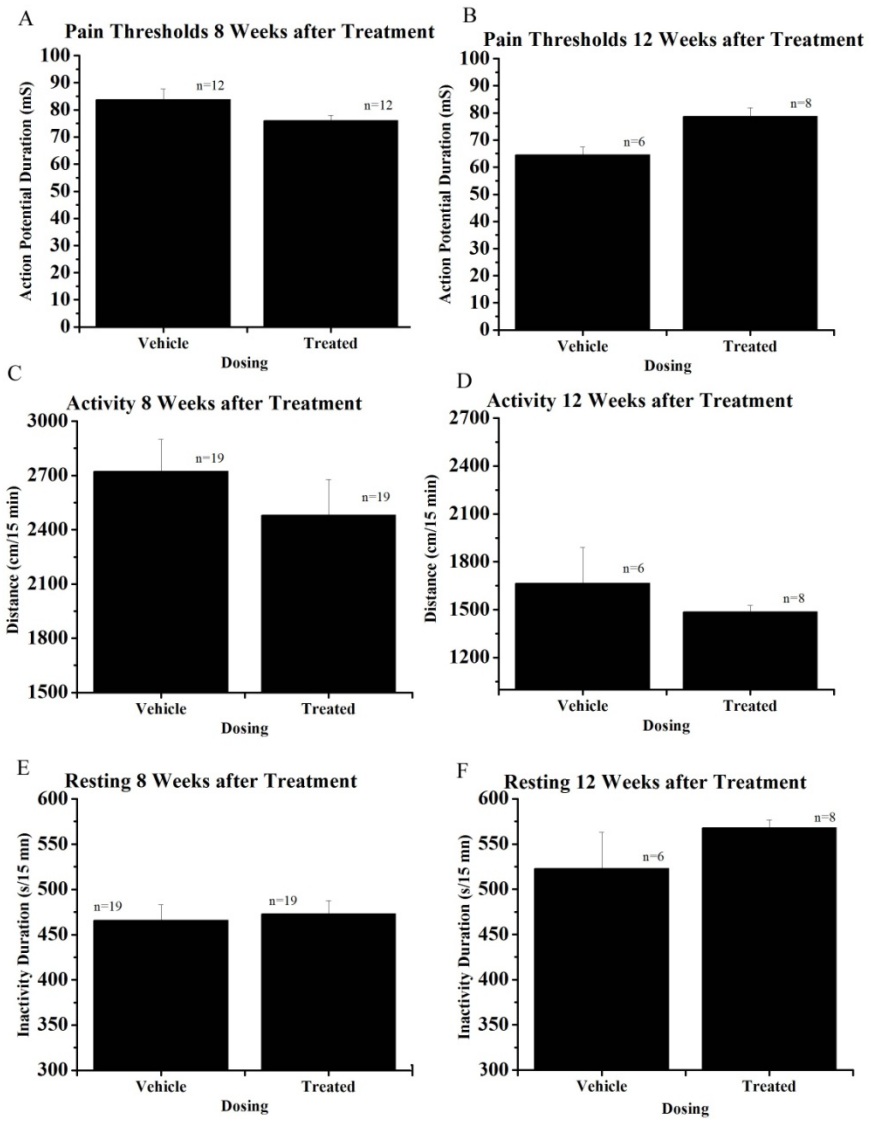

Figure 2. Pain and Activity Measures were Unchanged 8 and 12 Weeks after NTPB Exposure lasting 60 Days. There were no behaviors effects in the post treatment period.

Persistent Influences of Neurotoxicants and PB on Nociceptor Protein Function

Task 4: We will assess nociceptors for changes in K_v7 function that could contribute to chronic and widespread pain.

Task 2c: Assess Excitability in Muscle Nociceptors

Task 2d: Assess Excitability in Vascular Nociceptors

Task 4c: Assess Spontaneous Activity in Nociceptors

Task 4d: Assess the Nociceptor Phenotype Exhibiting Spontaneous Activity

GWI pain is often described by veterans as a widespread pain affecting muscle, joints and other deep tissues (Stimpson et al., 2006; Thomas et al., 2006). Accordingly, we focused our molecular studies on nociceptive neurons that innervated deep tissues with extensive bodily representation (muscles and vessels). Electrophysiological methods were used to examine the status of membrane proteins expressed in categories of skin, muscle and vascular nociceptors harvested from treated and control animals 4, 8 and 12 weeks after dosing had ceased (Rau et al., 2007; Rau et al., 2013).

Persistent Alteration in K^+ Channel Protein Function

K^+ currents were isolated as described in the Appendix (p. 73). The linopirdine sensitive K_v7 'tail' current was assessed across a series of deactivating voltages. In type 8 vascular nociceptors, significant decreases in the amplitude of the K_v7 currents were associated with treatment of rats with neurotoxicants in NR1, 30 days after the treatment had ceased ($F= 7.92$, $p<.002$; n=6,6; figure 3A). These decreases were significant at voltages near the resting membrane potential (-70, -50 mV; figure 4A), where activation of K_v7 currents are engaged in opposing neural discharge. This implies heightened excitability in this nociceptor family. In contrast, no differences were observed in muscle nociceptors (type 5) K_v7 function after NR1. This interesting K_v7 sensitivity to neurotoxicants was replicated following NR2 treatments (higher dosing). In both studies, the changes in K_v7 amplitude were specific to vascular nociceptors (figure 3). Although the K_v7 protein sensitivity to neurotoxicants was confirmed ($F=5.28$, $p<.03$, n=9,9), the polarity of the change *reversed* in NR2, where, with higher dosing, treated vascular nociceptive neurons manifested increased K_v7 current amplitude. Although the overall amplitude was increased, the voltage dependent changes in K_v7 amplitude were shifted to depolarized levels that would not oppose AP initiation (-30 and -40 mV; figure 4C). While the neurotoxicant action on K_v7 voltages were, to an extent, mixed, it is more important that we did observe, and replicated, that a neural protein was sensitive to neurotoxicant treatment; and that this sensitivity was unique to one class of tissue specific nociceptors and persisted beyond the immediate dosing period.

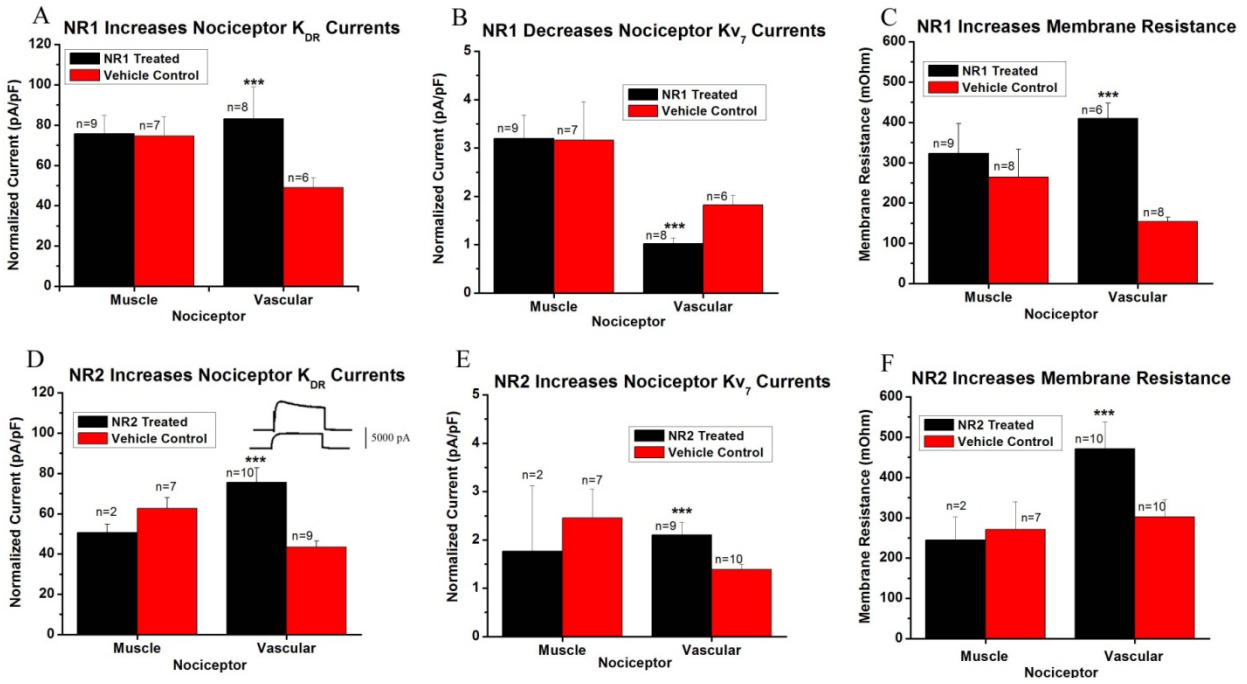

Figure 3. Neurotoxicants Altered K_v Protein Reactivity in Putative Vascular Nociceptors 4 Weeks After Treatment Ended. A-B) K^+ Channel proteins were modulated by NR1. **D-E)** The same K^+ channel proteins were modulated by NR2. Insert shows representative K_{DR} currents (upper: NR2 treated). **C and F)** Membrane resistance was increased by NR1 and NR2. Note the reversal of Kv_7 outcomes in 'B and E'. Na_v proteins were unaffected in skin, muscle or vascular nociceptor populations (not shown). *** significantly different from vehicle control.

In the course of assessing K_v7 currents we were able to simultaneously measure the total delayed rectifier currents (K_{DR}) present in muscle and vascular nociceptors. Although not part of the SOW, capture of this data was a natural consequence of the K_v7 experiments. K_{DR} currents are critical for directional stabilization of axonal conduction in axons. To our surprise, K_{DR} currents were substantially and significantly increased in vascular, but not muscle nociceptors, following both NR1 and NR2 treatments (figure 3B, E). Parallel increases in whole cell membrane resistance complicated the interpretation of this finding, as other K^+ current proteins that govern membrane resistance (K2p) could indirectly influence K_{DR} voltage reactivity (figure 3C,F). Distinct experiments are needed to unravel the relative contributions K2p and K_{DR} proteins.

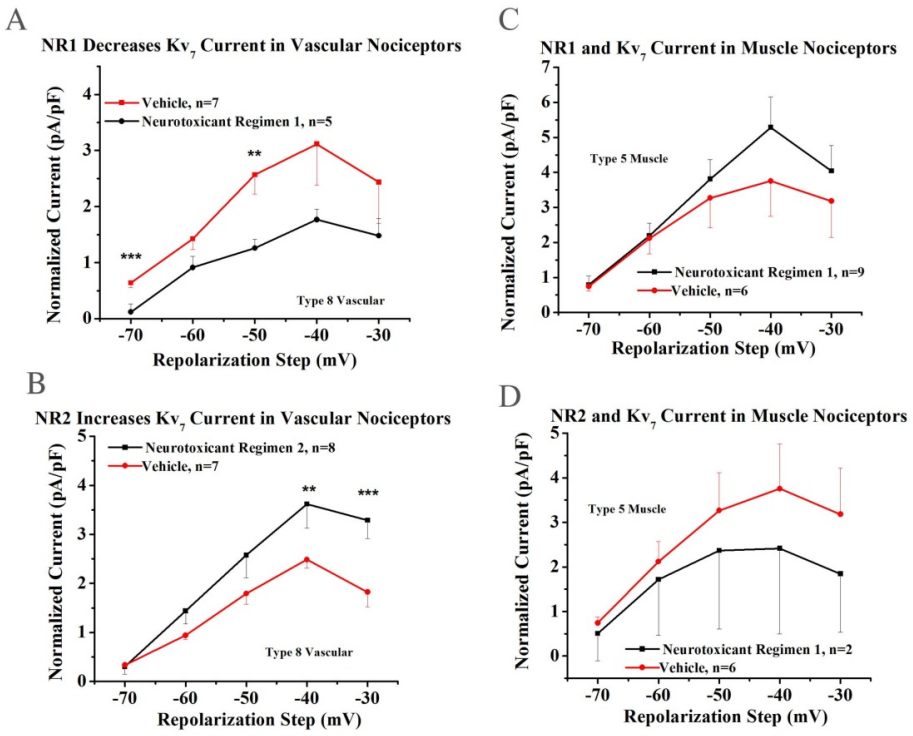

Figure 4. Neurotoxicants Alter Voltage Dependence of K_v7 in Vascular Nociceptors Following NR1 and NR2. A) K_v7 mediated currents are decreased near the resting membrane potential after NR1 (~-60 mV). **B)** Muscle nociceptor K_v7 was not shifted by NR1 treatment. **C)** K_v7 mediated currents are increased at depolarized voltages after NR2 (~-60 mV). **D)** There was insufficient muscle nociceptor K_v7 data for a statistical test. NR1: *** p< .003, ** p< .02; NR2: *** p <.009, ** p< .03. Bonferroni corrected for type 1 error.

During this performance period, we examined whether changes in K_v7 currents, following treatment at the higher dose and a longer exposure (60 days), would persist to 8 and 12 weeks delays after exposure to the neurotoxicants. Molecular tests conducted on K_v7 during NR3 (NTPB) were consistent with those in NR2 (same dose/30 day exposure). At the high dose, and with an extended exposure duration (60 vs 30 days), K_v7 currents in vascular nociceptors were increased at the 8 week delay period (figure 5). Although these increases subsided at the 12 week delay (figures 5 and 6) the outcome reinforced the notion that K_v7 currents might be an important key to understanding dysfunctional nociceptor activity underlying chronic pain conditions. Cellular studies conducted at the same dosing and timing as the molecular studies seemed to support the molecular data and the notion that K_v7 played an important role, and that vascular nociceptor were more sensitive to maladaptations caused by neurotoxicants than muscle or skin nociceptors.

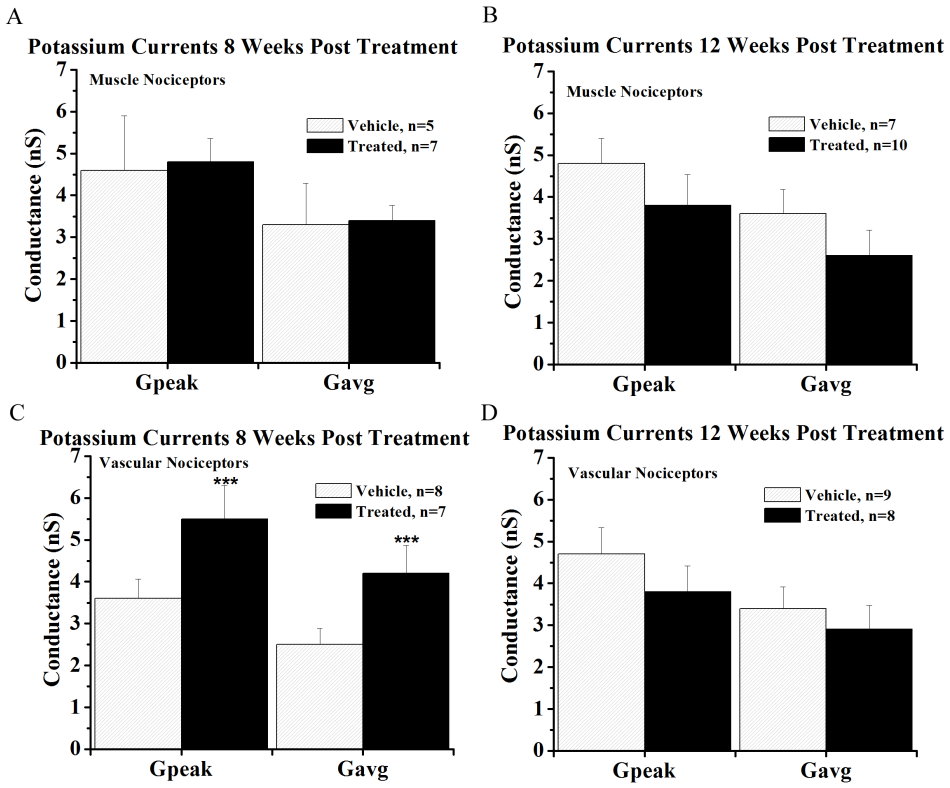

Figure 5. Peak and Average Conductance of K_V7 are Increased 8 Weeks after NTPB. A and B) K_V7 currents are unchanged in muscle nociceptors at the 8 and 12 week delay. **C and D)** K_V7 current are increased in the 8 week delay but return to normal at the 12 week delay in vascular nociceptors ($p<.05$ and $p<.05$ for peak and average respectively).

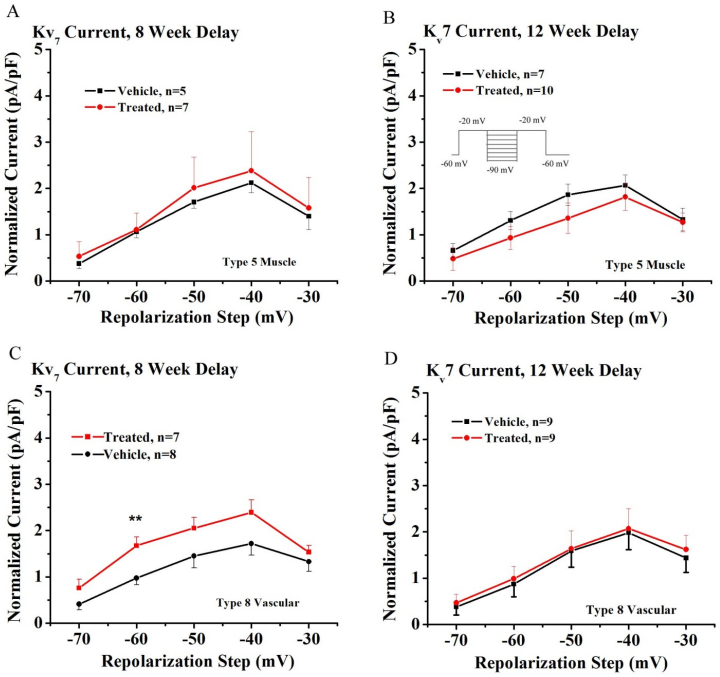

Figure 6. Voltage Dependence of K_V7 Current 8 and 12 Weeks Following 60 Days of NTPB. A and B) The voltage dependence of the normalized current was unchanged in muscle nociceptors. **C and D)** The voltage dependence of the normalized current was unchanged in vascular nociceptors. The normalized current was significantly increased at -60 mV ($p<.03$, C). The increase of the normalized current did not persist for 12 weeks (D).

Cellular Studies. Studies were carried out to determine the character and extent of functional changes to muscle and vascular nociceptors in NTPB and vehicle treated rats.

Muscle and vascular nociceptors from the 8 week delay group were brought into current clamp mode. Neurons were examined for the presence of any spontaneous activity at 22° and 35° C. Cellular tests were conducted 8 weeks after cessation of treatment, and corresponding to the time period at which we observed changes in channel physiology. We did not conduct any cellular tests at the 12 week delay.

We examined whether the NTPB treated neurons exhibited spontaneous activity (90 sec observation period). Spontaneous activity in nociceptors is very rare but would be expected in many chronic pain conditions where pain is present, but there is no obvious tissue damage. We made tests at room temperature (22° C) and at physiological temperatures (35° C) where the behavior of nociceptors might be altered by the higher current kinetics present at mammalian body temperature. Studies at body temperature are typically avoided in electrophysiology as they are difficult to perform and it is more challenging to maintain the health of neurons, in vitro, at the metabolic rates associated with body temperature.

At the 8 week delay period, spontaneous activity was observed in 3 treated vascular nociceptors at 22° C (41.3 +/- 25.8 APs; 3 of 11 cases). Vascular nociceptors in vehicle treated rats did not exhibit any spontaneous activity (n=10; see figure 7A). When the bath temperature was raised to 35° C, 6 NTPB treated vascular nociceptors exhibited spontaneous activity while only 2 vehicle treated vascular nociceptors discharged (59.2 +/- 28.1 vs 4.0 +/- 3.5 APs; n= 9 and 10 respectively; $p<.05$). However, 2 of the 6 vascular NTPB treated nociceptors discharged weakly (2 and 4 APs total). Muscle nociceptors were less reactive but did exhibit higher trends. Two of 6 muscle nociceptors discharged at 35° C (33 and 1 AP). None of the control muscle nociceptors discharged at 35° C (n=3). There was no activity at 22° C in muscle nociceptors.

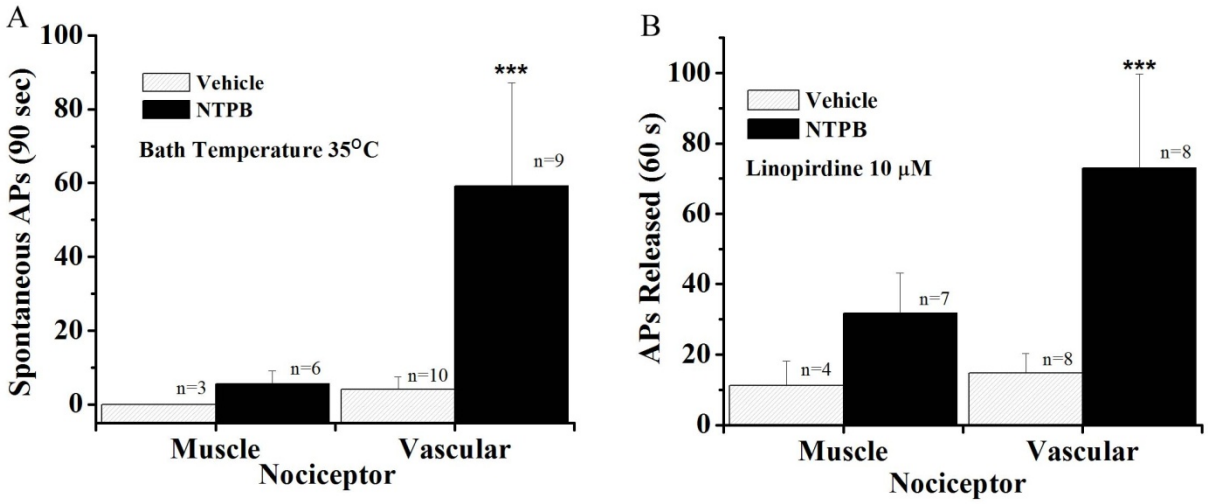

Figure 7. Spontaneous Activity increased in Vascular Nociceptors from NTPB Treated Rats.

Due to the importance of spontaneous discharge to GWI related pain, we conducted additional tests to examine the specific relationship between K_v7 and vascular nociceptors chronically exposed to NTPB. Following a 6 minute exposure to a specific inhibitor of K_v7 mediated current (linopirdine), we observed that action potentials were emitted spontaneously from both muscle and vascular nociceptors. The number of APs emitted by NTPB treated rats were considerably higher in both muscle and vascular nociceptors (figure 7B). The increase in spontaneous activity in type 8 vascular nociceptors was statistically significant relative to vehicle treated animals. Increases in spontaneous activity of muscle nociceptors failed to achieve significance ($p<.24$). These data suggest that, in the NTPB treated rats, K_v7 was suppressing a higher tendency for spontaneous AP discharge in vascular nociceptors. We repeated these tests at the 12 week observation interval when vascular nociceptor K_v7 currents had returned to normal levels. The six minute linopirdine treatment did not expose any heightened tendencies for spontaneous discharge at the 12 week delay. The factors potentiating discharge tendencies was not clear from this data, but could be due to Na_v currents, shown below, to have been modified at the 8 week delay by NTPB.

Chronic Neurotoxicant/PB Administration and Na_v Protein Function in Skin, Muscle and Vascular Nociceptors

Due to the demonstrated interactions between type I pyrethroids and Na_v proteins (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996a,b; Tabarean and Narahashi, 1998; Tabarean and Narahashi 2001) we hypothesized that long term exposure to permethrin and other neurotoxicants would produce persistent derangements in Na_v activity. Besides the actions of anticholinesterases, alterations of K^+ channel activity observed after NR1 NR2 and NR3 (NTPB) could reflect compensation for increased Na_v function. Accordingly, we examined $Na_v1.8$ and $Na_v1.9$ activation, deactivation and steady-state inactivation in rats treated with neurotoxicants/PB following a 30 day exposure to NR1 or NR2 or 60 day exposure to NR3 (NTPB).

Task 2: We will assess nociceptors for changes in Na_v function that could contribute to chronic and widespread pain following treatment with 3 neurotoxicants

Task 2c: Assess Excitability in Muscle Nociceptors

Task 2d: Assess Excitability in Vascular Nociceptors

Task 4c: Assess Spontaneous Activity in Nociceptors

Task 4d: Assess the Nociceptor Phenotype Exhibiting Spontaneous Activity

Studies on $Na_v1.8$. In year 2, we expanded studies of nociceptor $Na_v1.8$ physiology. The new studies included the influence of increased neurotoxicant exposure duration and increased delay after treatment (NTPB).

Nociceptors express the TTXr (tetrodotoxin resistant) $Na_v1.8$ protein (Djouhri et al., 2003a,b; Amir et al., 2006; Jiang et al., 2011) that are known to be acutely influenced by permethrin (Jiang et al., 2013), as well as other pyrethroids (Bradberry et al., 2005; Ray and Fry, 2006). Neurons were harvested from chronically treated rats (Table 1). Following isolation of $Na_v1.8$ in skin, muscle or vascular nociceptors (see Appendix p. 75), we applied protocols to characterize fundamental properties of voltage sensitive proteins (activation, inactivation and deactivation). Studies were conducted 4 and 8 weeks after dosing with neurotoxicants and PB had ended.

Activation reflects the tendency for $Na_v1.8$ protein to form action potentials and is an important gauge of excitability. These data were assessed by formation of classic sigmoidal activation curves (Boltzman functions) where a $V_{.50}$ activation statistic reflects the voltage at which half of the proteins are activated (also designated $V_{1/2}$). Inactivation was also assessed by classic methods. A $V_{.50}$ was derived that reflected the voltage at which half of the $Na_v1.8$ proteins were available to be activated. A depolarizing (more positive) shift of the inactivation $V_{.50}$ indicates that a larger portion of the total pool of $Na_v1.8$ proteins are capable of being activated. Deactivation refers to the movement of proteins from an open to a closed state due to a hyperpolarizing ion flux. The slowing of deactivation leads to greater excitability. Deactivation was assessed by deriving time constants (tau_{deact}) that characterized the exponential decay of $Na_v1.8$ mediated current in proportion to a series of deactivating voltage steps. Normalized peak currents and conductances were also assessed. Conductances are used to account for the influence of driving force (voltage) on current amplitude.

Although $Na_v1.8$ is greatly influenced by acute permethrin administration (see figures 21, 23 and 24, pages 27, 39 and 41), we did not observe a lasting influence on $Na_v1.8$ expressed in muscle or vascular nociceptors that had been *chronically* exposed to permethrin as a component of the neurotoxicant regimens NR1 and NR2. There was no indication that the tau_{deact} of $Na_v1.8$ was modified in either mean duration or voltage dependence 4 weeks after a 30 day exposure to permethrin, chlorpyrifos and PB (NR1, NR2; figure 8). Nor were changes in steady state inactivation apparent in any tissue specific nociceptor population examined (figure 9). Fits of exponential decay functions to the decay phase of the $Na_v1.8$ were also unaffected by NR1 or NR2 treatments (see figure 9 imbeds). With the exception of skin nociceptors, the normalized peak $Na_v1.8$ current was also unaffected (I_{max}/C_m; figure 10), as was, in limited studies, the voltage dependent activation (figure 11). Therefore, the acute influences of permethrin on $Na_v1.8$, demonstrated during 'Acute Studies' of Task 4 (see below, p. 31), and in some instances demonstrated with other type I pyrethroids (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996a,b; Tabarean and Narahashi, 1998; Tabarean and Narahashi, 2001) were not present 4 weeks after chronic exposure to permethrin and other suspect GWI agents.

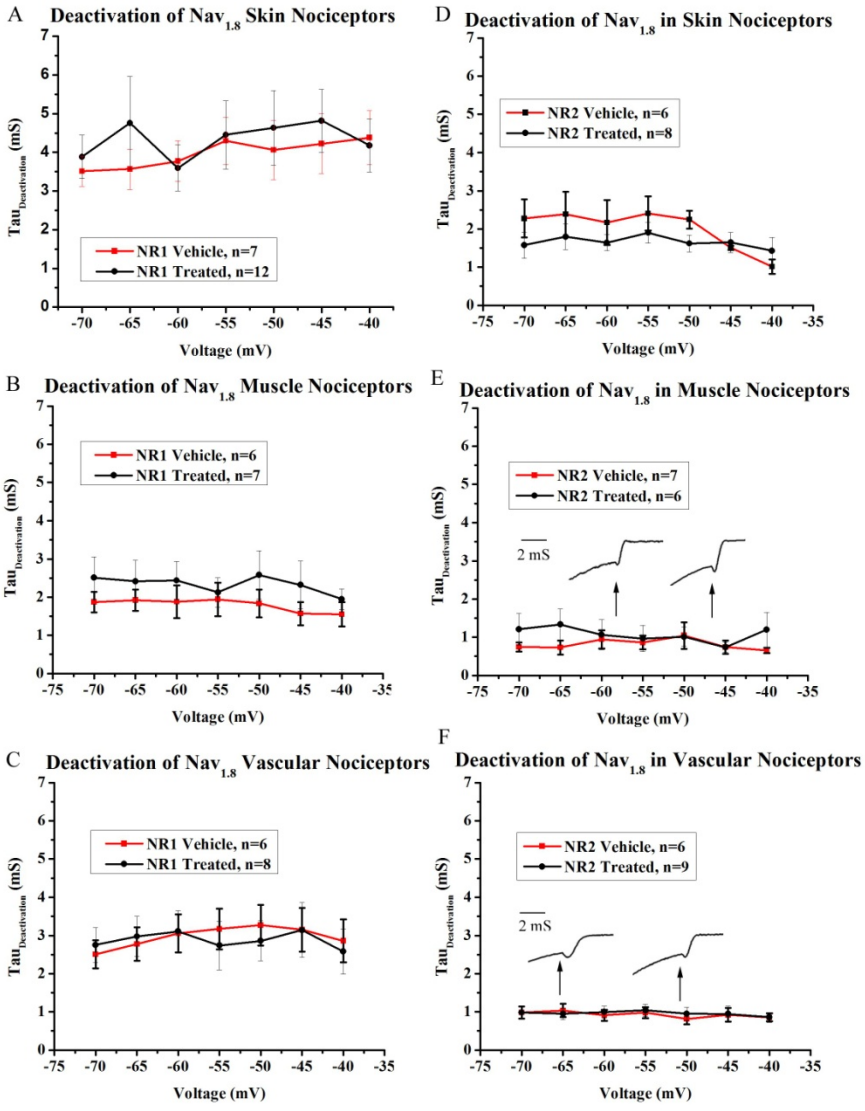

Figure 8. Dosing with NR1 or NR2 did not Modify $Na_v1.8$ Deactivation. A-C) Four weeks after NR1 dosing, there were no differences in the tau_{deact} in skin (A), muscle (B) or vascular nociceptors (C). **D-F)** Four weeks after NR2 dosing, there were no differences in the tau_{deact} of in skin (D), muscle (E) or vascular nociceptors (F). Inserts in E and F are truncated representative deactivation currents in muscle and vascular nociceptors (-40 mV). The arrow indicates the point of deactivation. The upper trace came from a vehicle exposed animal. The lower trace came from a neurotoxicant treated animal. The Nav current trace was greatly truncated to enable presentation of the deactivation tail current. See figure 23, p. 39 for a deranged tail current typical of acute permethrin treatment.

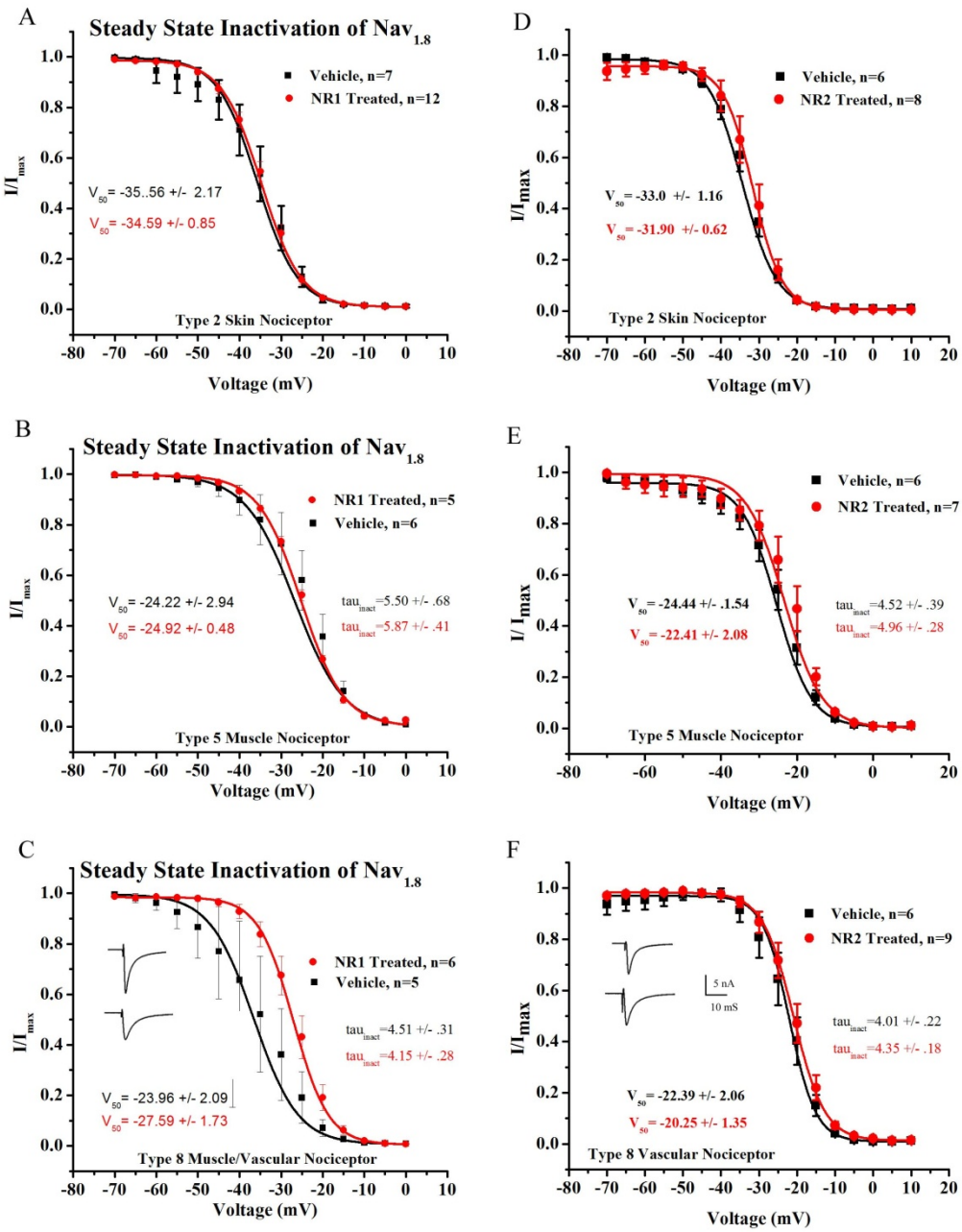

Figure 9. Dosing with NR1 or NR2 did not Modify $Na_v1.8$ Inactivation. A-C) Four weeks after NR1 dosing, there were no differences in the V_{50} of steady state inactivation in skin (A), muscle (B) or vascular nociceptors (C). **D-F)** Four weeks after NR2 dosing, there were no differences in the V_{50} (mV) of steady state inactivation in skin (D), muscle (E) or vascular nociceptors (F). Inserts in (C) and (F) are representative $Na_v1.8$ currents in vascular nociceptors (0 mV). The upper trace came from a vehicle exposed animal. The lower trace came from a neurotoxicant treated animal. Scale bars in 'F' apply to 'C'. Calculated tau_{inact} (mS) is shown as an insert in plots of muscle and vascular nociceptors. There were no significant differences in treatment conditions.

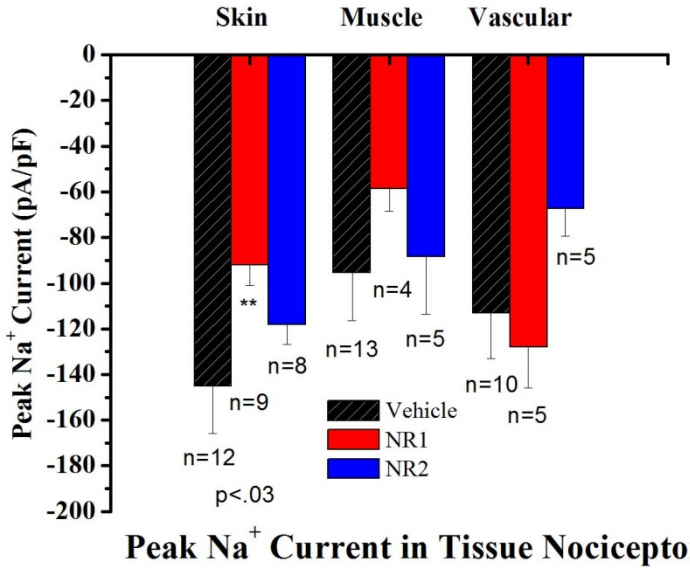

Figure 9. Peak Na^+ Currents and Neurotoxicants. Mainly decreasing trends were observed in peak $Na_v1.8$ currents in tissue specific nociceptors. A significant decline in skin nociceptors (**) was observed following NR1 only but it was not replicated in NR2. It was non-significant with a Bonferroni correction for type 1 error.

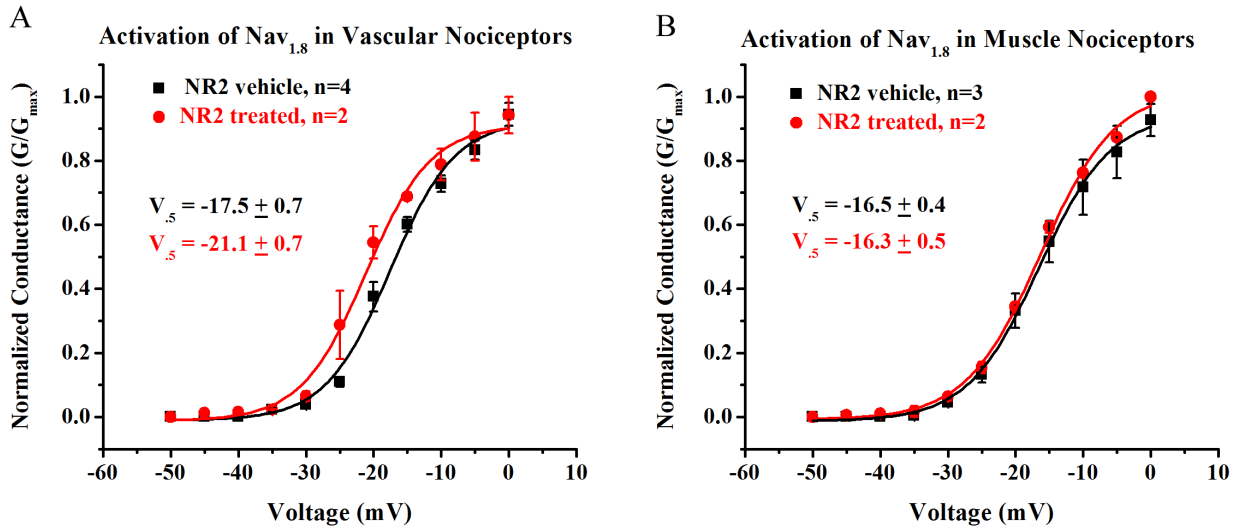

Figure 10. Dosing with NR2 did not Modify $Na_v1.8$ Activation. A) The $V_{.5}$ for putative vascular nociceptor activation was shifted, but insufficient data was obtained for a statistical test. **B)** The $V_{.5}$ of muscle nociceptors exhibited little neurotoxicant influence 4 weeks after NR2 treatment.

When we extended the duration of exposure to 60 days (NTPB group), we began to detect discrete influences of chronic exposure to NTBP on $Na_v1.8$ physiology. Although there were few demonstrable effects on protein physiology normally associated with the acute influence of permethrin we did observed shifts in the kinetics on $Na_v1.8$ with NTPB exposure. The details are presented below.

TTXr $Na_v1.8$ is powerfully modified by acute permethrin administration. In this report, the permethrin sensitive properties of $Na_v1.8$ were largely unaffected by a 60 day exposure to permethrin and the other agents. While acute permethrin produces >10 mV hyperpolarizing shift in $V_{1/2}$, no lasting influence on voltage dependence was observed after chronic exposure to NTPB (figure 11); and in contrast to well established acute effects on the kinetics of deactivation (~2 fold increase; Jiang et al., 2013), there was no indication that the tau_{deact} of $Na_v1.8$ was modified in either mean duration or voltage dependence 8 weeks after a 60 day exposure to permethrin and other agents (figure 12).

In contrast, $Na_v1.8$ decay constants (tau_{decay}), which are typically lengthened by pyrethroids, (Jiang et al., 2013) remained elongated 8 weeks after administration of the neurotoxicant cocktail (figure 11 inserts). We assessed these decay constants from both activation (-20 mV test) and steady state inactivation protocols (10 mV test). In both of these independent analyses (separate cells), the tau_{decay} values were significantly lengthened for muscle nociceptor $Na_v1.8$. No changes were observed in vascular nociceptors tau_{decay} following NTPB exposure. Despite the slowing of decay, the normalized peak $Na_v1.8$ current was not affected in muscle or in vascular nociceptors (I_{max}/C_m; data not shown). We did not examine $Na_v1.8$ physiology 12 weeks after treatment.

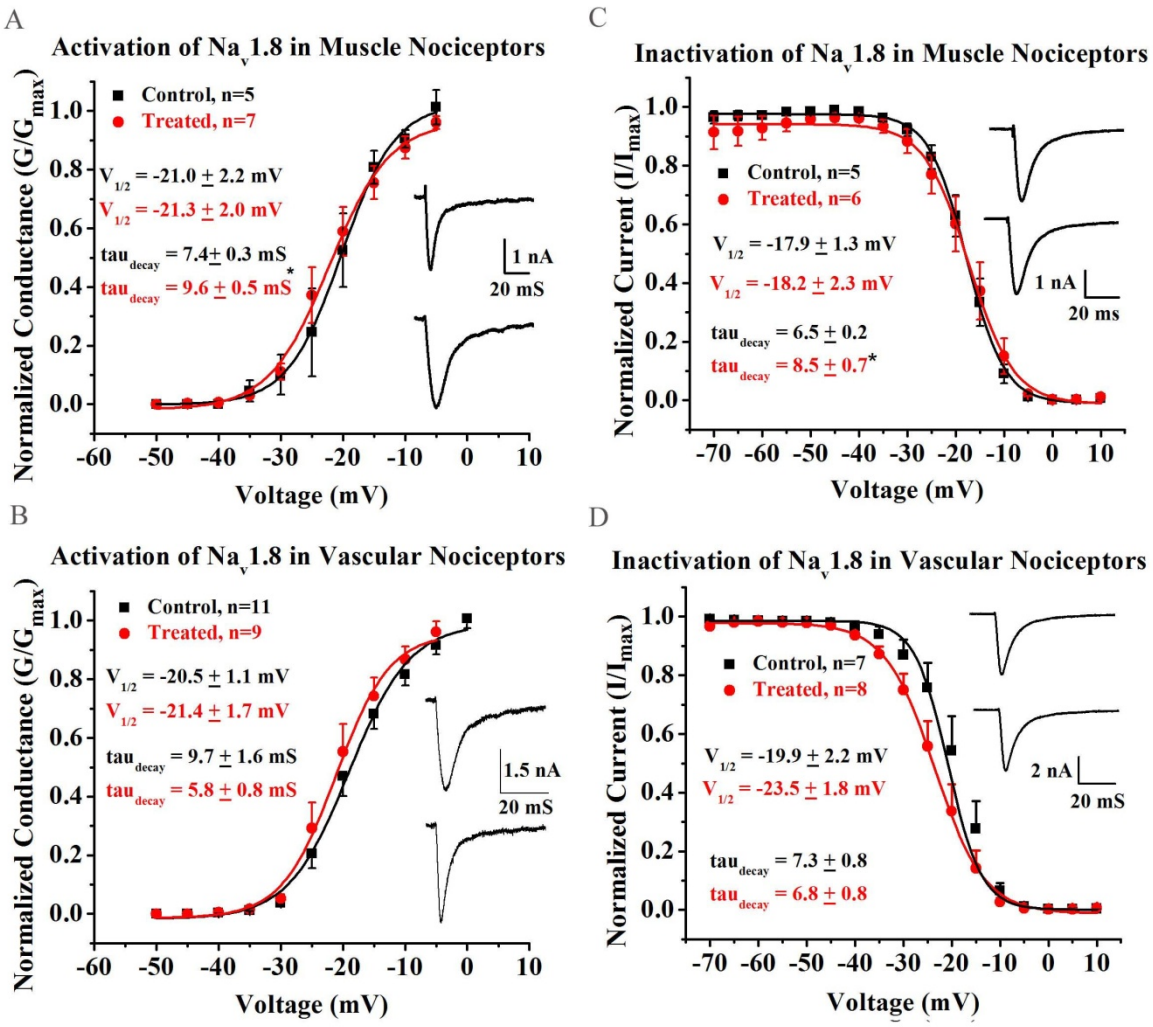

Figure 11. Voltage Dependent Activation and Inactivation are Unchanged by NTPB Treatment. A and B) Muscle nociceptor activation and inactivation $V_{1/2}$ remained stable 8 weeks after a 60 day exposure to NTPB. Decay kinetics were signficantly longer in both tests (see insert taudecay; p<..02 and p<.04, A and B respectively). Representative traces are presented. **C and D)** Vascular nociceptor activation and inactivation $V_{1/2}$ remained stable 8 weeks after a 60 day exposure to NTPB. Decay kinetics were not altered (insert taudecay). Representative traces are presented (vehicle upper; NTPB lower).

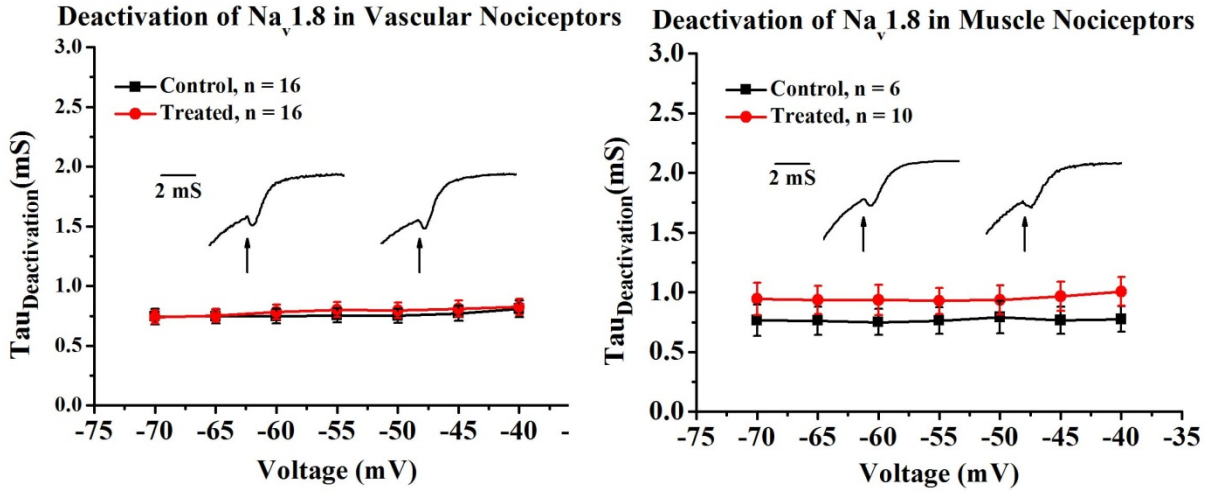

Figure 12. Mean and Voltage Dependent Kinetics of Deactivation are Unchanged 8 Weeks after Treatments. Left panel: Vascular Nociceptors; **Right Panel:** Muscle nociceptors. Inserts present representative deactivation traces that are greatly shortened for visualization. Arrows indicate deactivation step.

Cellular Studies at the NR1 Dose Level. Action potentials represent voltage shifts resulting from the net current flow of many membrane proteins (Na_v, K_v, Ca_v, K_{ca}) that are expressed in multiple forms and differentially expressed across nociceptor phenotypes. As such, changes in the shape or excitability of the action potential represents changes in the contribution of these many proteins. We determined whether NR1 produced any shifts in action potential features 4 weeks after NR1 administration.

Neurons were harvested from rats receiving vehicle or NR1 treatment. Nociceptors were characterized as muscle or vascular nociceptors and brought into current clamp mode. The resting membrane potential was adjusted to -60 mV and action potentials (3) were evoked by current injection (3-5 nA; 1 msec). Action potential features were assessed; including action potential duration (APD) and afterhyperpolarization duration (AHD; Petruska et al, 2000; Petruska et al., 2002). Excitability was subsequently determined by stepwise current injection, and the total number of APs evoked by 15 consecutive current injection steps were tallied (see Appendix, p. 71).

Contrasts performed between vascular and muscle nociceptors harvested from NR1 treated and vehicle treated rats did not reveal any changes in action potential duration (APD; figure 13A), afterhyperpolarization (AHD; figure 13B), resting membrane potential (figure 13C) or

excitability in muscle or vascular nociceptors (figure 13D and E). Although trending higher, there were no changes observed in or membrane resistance (figure 13F). The pattern of changes were similar after NR2 (figure 13). Following tests on excitability, cells from rats exposed to NR1 or NR2 ells were observed for the presence of spontaneous activity (2.5 min). There were no significant differences in spontaneous activity between neurotoxicant and vehicle treated rats, and little spontaneous activity was observed in any nociceptor class after NR1 (tests conducted at 35° C; data not shown).

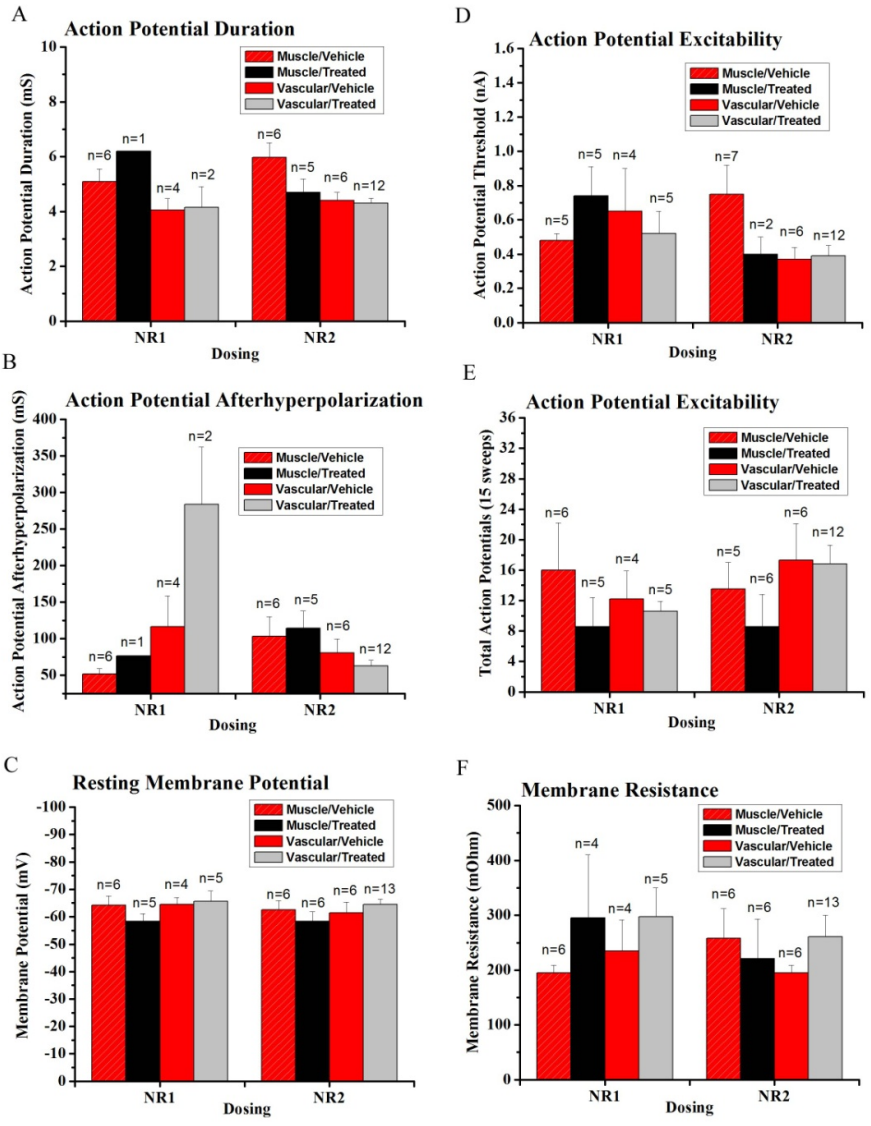

Figure 13. Neurotoxicant Treatments and Action Potential Features. A-C) Action potential features remained stable in muscle and vascular nociceptors after NR1 and NR2. **D-E)** Action potential excitability (threshold and evoked APs) were unaffected by neurotoxicant treatments. **F)** Although membrane resistance was consistently higher, there were no significant effects on membrane resistance in any nociceptor class.

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Studies on $Na_v1.9$.

Task 2: We will assess nociceptors for changes in Na_v function that could contribute to chronic and widespread pain following treatment with 3 neurotoxicants

For studies involving Na_v proteins, we will examine muscle, vascular and skin nociceptors that express $Na_v1.8$ and $Na_v1.9$

Task 2a: Assess Na_v Physiology in Muscle Nociceptors: 14 months (8-22)

Task 2b: Assess Na_v Physiology in Vascular Nociceptors: 14 months (8-22)

Task 2c: Assess Excitability in Muscle Nociceptors: 14 months (8-22)

Task 2d: Assess Excitability in Vascular Nociceptors: 14 months (8-22)

It has been demonstrated that many pyrethroid insecticides will modify the physiology of $Na_v1.8$ at room temperature. We have shown, in our studies, that acute permethrin produces substantial changes in nociceptor $Na_v1.8$, and that these influences persist at mammalian physiological temperatures (Jiang et al., 2013). The persistence at physiological temperature is an important finding because it has been long assumed that pyrethroids were ineffective at body temperature and therefore safe for humans. Yet with the ability of permethrin to modify Na_v physiology at body temperature, though greatly diminished in degree, gives cause to see how a chronic exposure to NTPB might induce long lasting maladaptations in $Na_v1.8$ or the channel proteins that oppose its heightened activity. As designated in the SOW, we broadened our investigation of the influence of permethrin and other agents on the physiology of another voltage activated Na^+ channel, $Na_v1.9$.

Although the influence of permethrin on Na_v has been studied for many years, new forms of Na_v have been discovered relatively recently ($Na_v1.9$; Tate et al., 1998; Cummins et al., 1999). $Na_v1.9$ is unique from all other Na_v in that Na_v 1.9 exhibits slow activation kinetics and ultraslow decay kinetics. Moreover, unlike any other Na_v, $Na_v1.9$ cannot, by itself, form an action potential. Rather, it is believed that $Na_v1.9$ potentiates sub-threshold currents from other sources to bring them to levels that will trigger AP forming Na_v like $Na_v1.6$, $Na_v1.7$ and $Na_v1.8$. $Na_v1.9$ is also unique in that it is expressed exclusively in nociceptors (Fang et al., 2002). Because of its limited distribution, nothing is known about the influence of pyrethroids on $Na_v1.9$. As part of Task 2 of the SOW, we examined the influence of chronic exposure of NR3 (NTPB) on the physiology of $Na_v1.9$ expressed in muscle and vascular nociceptors. Studies were made on

neurons harvested from rats 8 and 12 weeks after a 60 day exposure to the NR3/NTPB regimen (Table 1, p. 6). Studies investigating the acute influence of permethrin on $Na_v1.9$ are also currently underway as part of the EWOF.

Although the changes we observed in $Na_v1.8$ were relatively modest, substantial and relatively persistent changes in $Na_v1.9$ physiology followed from a 60 day exposure to neurotoxicants. When cells were harvested at the 8 week delay, we recorded significant increases in the peak and average conductance of $Na_v1.9$ in NTPB exposed rats (figure 14). Increases were significant for both muscle and vascular nociceptors. The kinetics of activation (tau_{act}) were also significantly modified in NTPB treated rat nociceptors (figure 16). There was no change in the voltage dependence of activation or deactivation; although, the kinetics of deactivation was significantly faster in vascular nociceptors (figure 17).

Studies were repeated at the 12 week delay. A portion of the NTPB influences on $Na_v1.9$ physiology were sustained at 12 weeks in vascular nociceptors (figure 15). In these studies, only vascular nociceptor data was collected in sufficient quantity to make a determination of the status of $Na_v1.9$ at this delay point. The peak and average conductance of vascular nociceptor $Na_v1.9$ remained considerably increased relative to vehicle treated animals; however, neither the peak conductance nor the average conductance quite reached significance ($p< .09$ and $p<.06$; figure 15). Nevertheless, consistent with the 8 week delay group, the tau_{act} of $Na_v1.9$ remained significantly slowed due to the chronic treatment with NTPB some 12 weeks earlier (figure 16B and D).

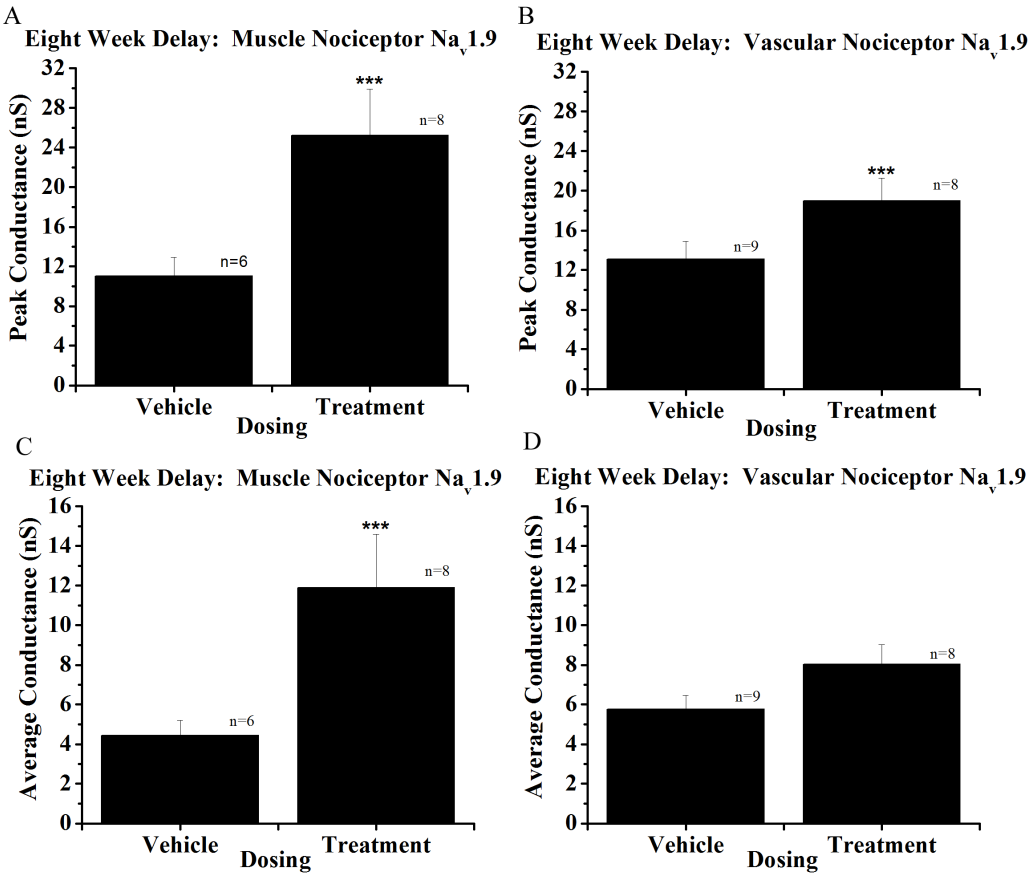

Figure 14. The Peak and Average Conductance of $Na_V1.9$ was Altered by NTPB. A and B) The peak conductance was significantly increased in muscle and vascular nociceptors ($p<.03$ and .05, A and B respectively). **C and D)** The average conductance (-70 to -40 mV) was significantly increased in muscle nociceptors and approached significance in vascular nociceptors ($p<.04$ and .07), A and B respectively).

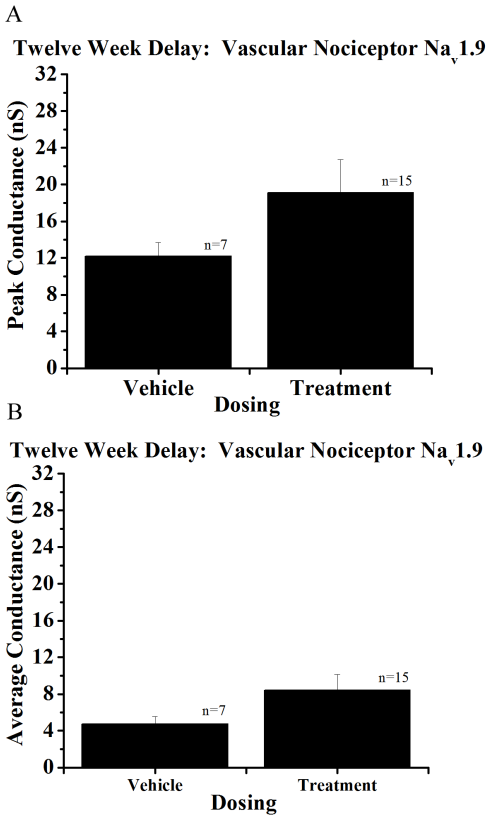

Figure 15. The Peak and Average Conductance of $Na_v1.9$ was not Altered by NTPB 12 Weeks after Treatment. A) Peak conductance increased and was close to significance ($p<.10$). **B)** Average conductance increased and was very close to significance ($p<06$). Only vascular nociceptors were examined in sufficient number at the 12 week delay to permit a statistical test.

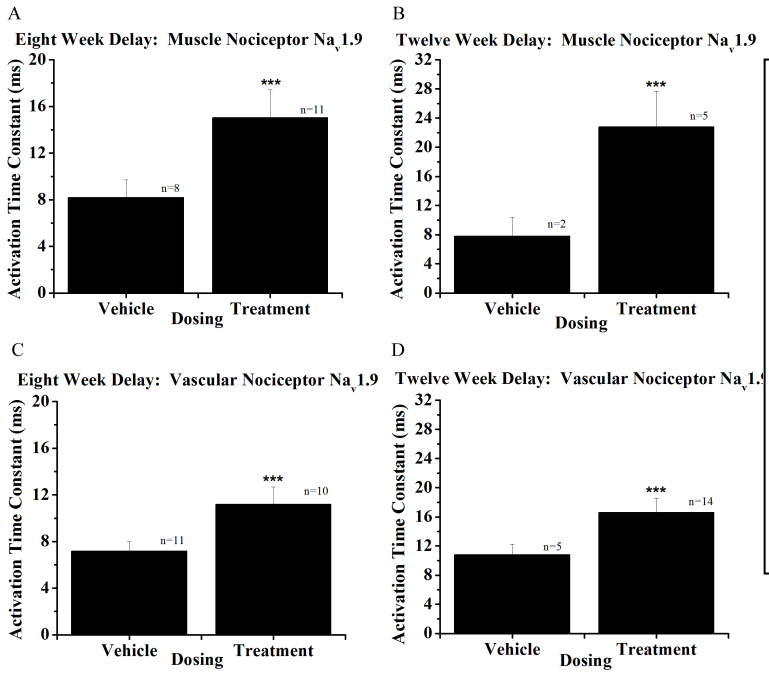

Figure 16. Activation Time Constants of $Na_v1.9$ were Increased 8 and 12 Weeks after NTPB. A and B) The kinetics of activation of muscle nociceptor $Na_v1.9$ are slowed 8 and 12 weeks after treatment with NTPB. The slowing was significant at 8 weeks but there was insufficient data at 12 weeks for a test ($p<.04$). **C and D)** The kinetics of activation of vascular nociceptor $Na_v1.9$ are slowed 8 and 12 weeks after treatment with NTPB ($p<.03$ and $p<.03$, A and B respectively).

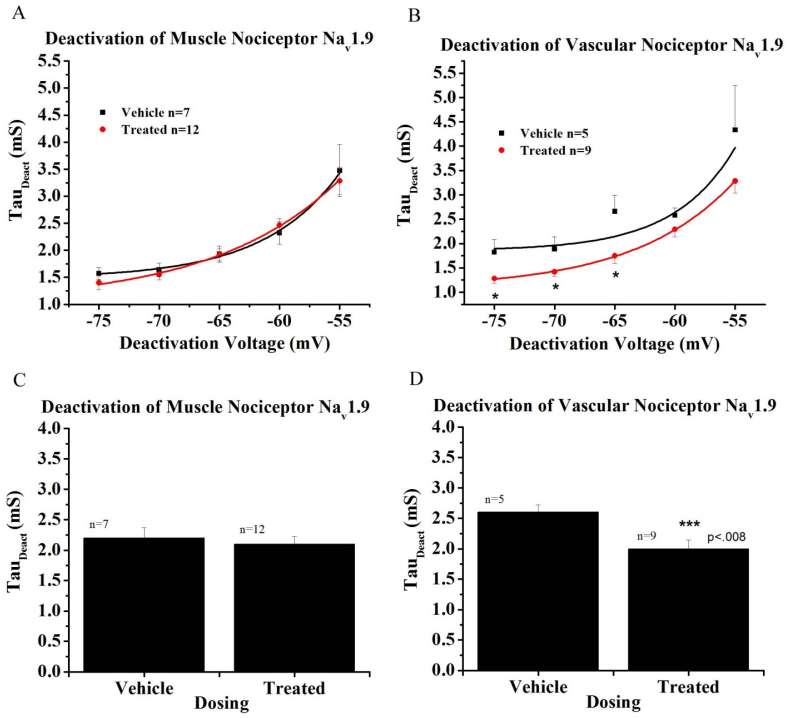

Figure 17. The Voltage Dependence of Deactivation Kinetics and Average Deactivation Kinetics after NTPB. A and B) The voltage dependence of muscle and vascular nociceptor deactivation is unaffected by NTPB at the 8 week delay. The kinetic rate was increased at -75, -70 and -65 mV for vascular nociceptors only (B). **C and D)** The average time constant of deactivation over the entire range tested was significantly reduced for vascular nociceptors (D).

We conducted cellular studies at the 8 week delay period to determine whether functional correlates could be found for these molecular adaptations to NTPB exposure. Studies were conducted as described on (p. 14). In contrast to studies using NR1 or NR2 dosing levels at 30 days, the NR3 dosing at 60 days produced functional changes AP characteristics. Despite the fact that NTPB induced changes in both muscle and vascular nociceptor $Na_v1.9$, all of the functional changes in nociceptor AP characteristics were limited to type 8 vascular nociceptors. This is not surprising as functional effects reflect the interaction of many different channel proteins. The functional impact of one maladapted protein expressed in a vascular nociceptor may be lessened due to a different constellation of channel proteins in a muscle nociceptor.

In vascular nociceptors, both the AP and AHP duration were significantly increased in the NTPB group (figure 18). There were no changes in the current injection excitability or threshold. It is possible that the increase in AP duration was due to the increased amplitude of $Na_v1.9$; but this

cannot be determined with any certainty from these experiments. Regardless, the increase in AP duration may be highly significant due to its implications for neuroplasticity. It is also possible that the increase in amplitude of $Na_v1.9$ is the driving current behind the spontaneous discharge we observed in the experiments of figure 7 (35° C and linopirdine released activity). We address these considerations more fully in 'Conclusions'.

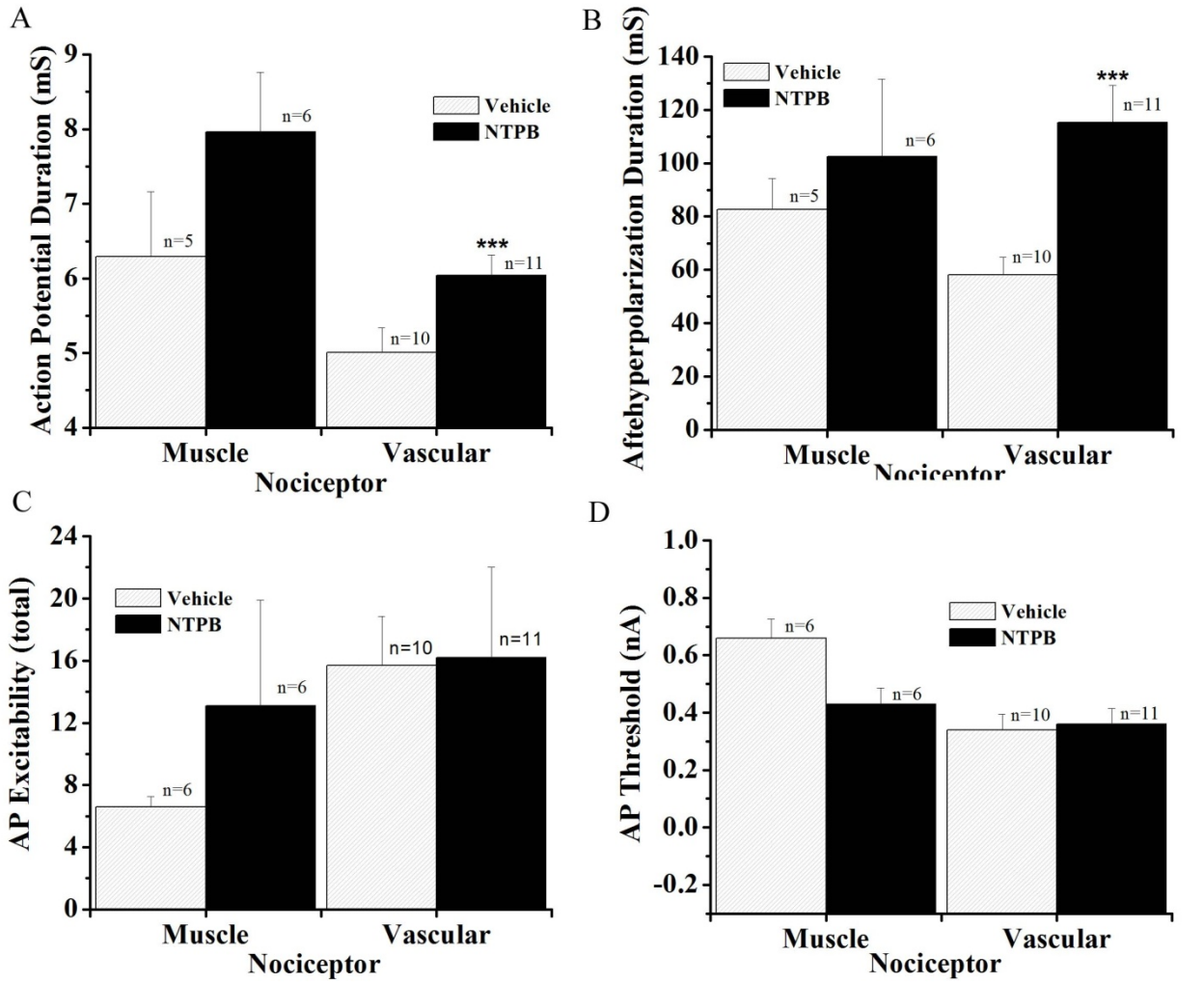

Figure 18. Action Potential Characteristics After a 60 Day Exposure to NTPB. Eight weeks following treatment some AP properties were significantly altered. **A)** Action potential duration significantly increased in vascular nociceptors ($p<.04$). **B)** Action potential afterhyperpolarization was significantly increased in vascular nociceptors ($p<.004$). **C and D)** No changes were observed in AP excitability or threshold.

Acute Influences of Permethrin on Skin, Muscle and Vascular Nociceptors

During the brief course of the Gulf war, GW veterans were exposed to 13 or more pesticides (DOD Environmental Exposure Report: Pesticides, 2003). Some of these pesticides have direct interactions with the pain system (pyrethroids: permethrin, phenothrin). The pyrethroid class of insecticides alters activity of mammalian proteins that are closely related to insect proteins by which pyrethroids exert their lethal effects (voltage gate sodium channels, VGSC, or Na_v). One of these mammalian proteins (i.e., $Na_v1.8$) is prominent in nociceptor (pain) coding (Djouhri et al., 2003; Jiang et al., 2011). Investigations of pyrethroid interactions with mammalian sensory neurons of the DRG (dorsal root ganglion), confirmed that powerful influences between several pyrethroids (allethrin, tetramethrin, deltamethrin) and sensory perceptions were possible (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996a,b; Tabarean and Narahashi, 1998; Tabarean and Narahashi 2001). While stationed in the Gulf region, GW veterans made liberal use of the type 1 pyrethroid insecticide permethrin. However the specific interactions between permethrin and peripheral nervous system nociceptors and/or functional $Na_v1.8$ channels expressed in these nociceptors is not known. Moreover, there are important functional differences between nociceptors innervating superficial (skin) and deep tissues (e.g., muscle) that could qualitatively affect their interaction with neurotoxicants. Accordingly, we initiated a series of studies to examine how permethrin interacts with specific skin, muscle and vascular nociceptors that express pyrethroid sensitive protein $Na_v1.8$, and other nociceptor proteins that might contribute to a chronic pain disorder.

As will be shown below, the acute influence of permethrin on nociceptor Na_v was extensive, and while affecting skin, muscle and vascular nociceptors alike, was especially potent and more pervasive in type 5 muscle nociceptors. While all the influences of permethrin on nociceptors was greatly diminished at mammalian physiological temperature, residual effects remained and could be a basis for chronic perturbations, which over time, could lead to neuronal maladaptations.

Task 3: We will examine the acute effects of permethrin on Na_v proteins in skin, muscle and vascular nociceptors

Studies on $Na_v1.8$. A series of studies on tissue specific nociceptor populations indicated that permethrin had broad effects on nociceptors, with additional influences specific to muscle nociceptors. Isolated nociceptors were characterized and identified as skin (type 2), muscle (type 5) or putative, type 8, vascular nociceptors (Appendix, p. 69; Rau et al., 2007; Rau et al., 2013). Cells were brought into current clamp mode and the influence of permethrin (10 μM) on nociceptor action potentials was assessed. As previously demonstrated in mammalian sensory neurons using the type II pyrethroid, deltamethrin (Tabarean and Narahashi, 1998), the type I pyrethroid, permethrin, significantly increased action potential duration of skin, muscle and vascular nociceptors (figure 19). The increase in duration was substantial and so extensive as to completely obscure the afterhyperpolarization period (6.96 +/- 0.8 vs 163.7 +/- 44.2; 8.8 +/- 0.7 vs. 105.9 +/- 12.2, and 6.9 +/- 0.6 vs. 82.7 +/- 12.4 ms for types 2, 5 and 8; $p<.001$, n=4-6; figure 19D, E, F). The afterhyperpolarization (AHP; figure 1b) opposes repetitive discharge and plays a critical stabilizing role in axonal transmission by preventing the occurrence of ectopic discharge that is believed to contribute to certain chronic pain syndromes (Devor, 2006; Baron, 2006).

We assessed whether permethrin-induced changes in AP characteristics would be associated with altered action potential excitability. Holding the cell at -60 mV to remove resting membrane potential biases on excitability, a series of current injections were performed on ETOH and permethrin treated nociceptors (10 current injection steps of 0.1-1.0 nA; 225 ms/test). Over an observation period of 10 minutes (1 min test intervals), the number of action potentials evoked by a series of current injections were increased in type 5 muscle nociceptors (13.0 +/- 4.5 APs; n=5; figure 20) but not in skin or vascular nociceptor classes. Often excitability tests on type 5 muscle nociceptors did not last the full 10 min period due to the development of spontaneous activity. Permethrin is known to produce depolarization and spontaneous activity in mammalian neurons in various species and preparations (Carlton 1977; Parkin and Le Quesne 1982, Takahashi and Le Quesne 1982; Staatz-Benson & Hosko, 1986; Meyer et al., 2008); however the instigation of spontaneous activity is not a universal property of type I pyrethroids like permethrin (Wright et al., 1988; Ray and Fry, 2006). This is the first demonstration that direct activation of nociceptors is possible and that a specific subclass of nociceptors exhibits distinct vulnerability to this neurotoxicant.

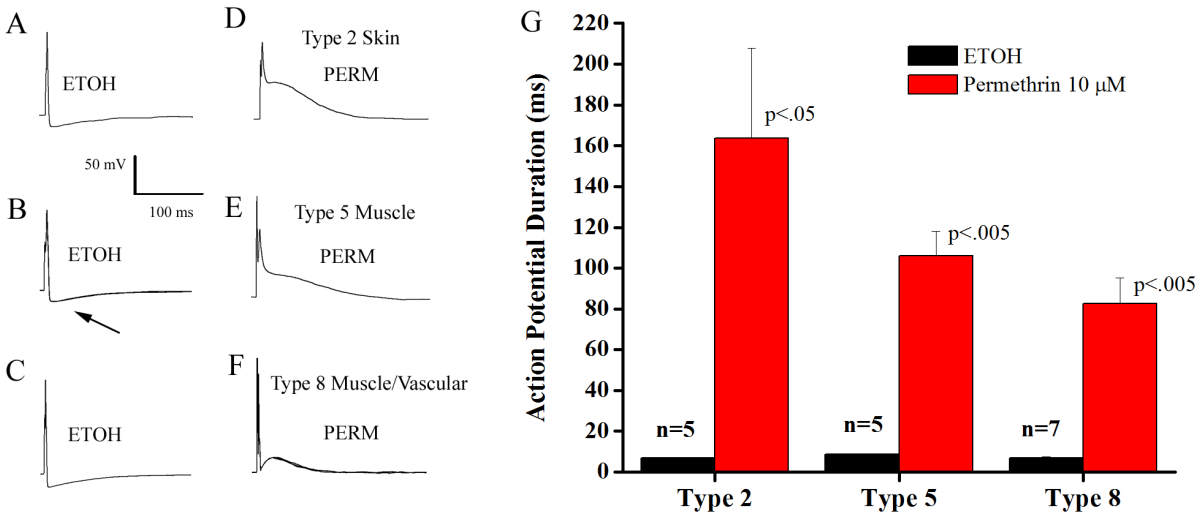

Figure 19. Permethrin Increases AP Duration in Nociceptors. A-C) Action potentials in tissue specific nociceptors following exposure to ETOH vehicle. The arrow highlights the afterhyperpolarization. **D-F)** Action potentials in the same nociceptors following exposure to 10 μM permethrin for 6-10 minutes. After-hyperpolarizations are absent in all treated neurons. **G)** Summary data for 3 nociceptive classes. Duration increases were highly significant. Action potentials were evoked by a 3 nA injection for 1 ms.

With the application of permethrin, all type 5 muscle nociceptors depolarized and exhibited spontaneous discharge (34.4 +/- 12.2 APs/5 min permethrin vs. 0.0 APs/5min ETOH; figure 20). In contrast, despite gradual depolarization of all cells in these conditions, neither vascular nor skin nociceptor populations exhibited spontaneous activity following permethrin application (n=16). ETOH vehicle did not produce spontaneous activity in any nociceptor class over equivalent application periods (n=8). It is noteworthy that depolarizations induced by permethrin exceeded those associated with ETOH vehicle alone (-6.98 +/- 2.2 vs. -17.5 +/- 2.7 mV in 5 min; n= 6 and 11 (TTX cases included)). This was consisted with a distinct contribution of permethrin to the shift in resting membrane potential to depolarized levels sufficient to initiate spontaneous activity. We initiated studies to identify the molecular basis of spontaneous activity in muscle nociceptors.

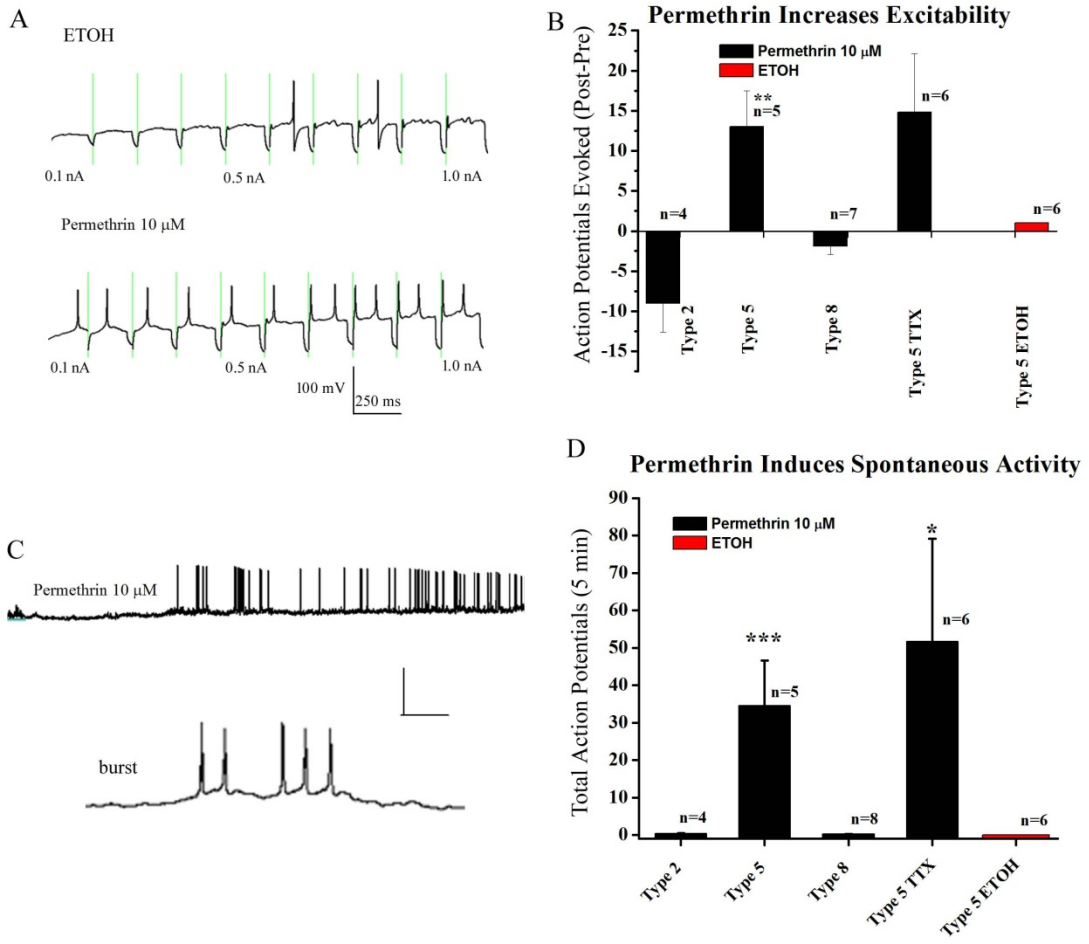

Figure 20. Permethrin Increases Excitability and Induces Spontaneous Activity in Muscle Nociceptors. A) Excitability testing in a type 5 muscle nociceptor. Ten consecutive current injection tests in an ETOH (upper panel) and Permethrin treated case (lower panel; 10 μM, 10 min). Vertical green bars separate consecutive tests. Substantial increases in action potential discharge were evident. **B)** Summary data for excitability testing indicated that only type 5 muscle nociceptors manifested excitability shifts. TTX did not block shifts in AP excitability (** p<.03 vs ETOH). **C)** Representative spontaneous activity in a type 5 muscle nociceptor after exposure to permethrin (10 μM). Five minutes of observation are shown. Exposure to ETOH for 5 minutes did not result in spontaneous activity (not shown). **D)** Summary data of the influence of permethrin on spontaneous activity. Only type 5 muscle nociceptors exhibited spontaneous activity. TTX did not impair the development of spontaneous activity. * p<.03 vs ETOH (Mann-Whitney U test; ties were not corrected). *** p<.001

Voltage gated Na^+ channels are routinely divided into families of proteins that are broadly distinguished by their sensitivity to the puffer fish toxin, TTX (tetrodotoxin). To examine whether TTXs (TTX sensitive) currents were essential to the development of spontaneous activity, we treated a group of type 5 muscle nociceptors to a combination of permethrin and TTX (10 and 1 μM; n=6). Using identical procedures as above, 5 of 6 TTX treated nociceptors depolarized and became spontaneous in the presence of permethrin+TTX (51.6 +/- 27.5

APs/5min; n=6; figure 20D). All TTX treated cases also exhibited increased excitability (figure 20B); action potential duration was not influenced co-application of TTX (105.9 +/-12.2 vs. 115.0 +/- 6.1 ms with TTX (not shown). We can conclude from these studies that TTXr ($TTX_{resistant}$) Nav proteins, such as $Na_v1.8$, were sufficient to maintain permethrin influences on AP duration, excitability and spontaneous activity of muscle nociceptors.

The action potential represents a transient disturbance in the net flow of ions across the neural membrane. The flow of ions is governed by the opening and closing of several families of voltage gated proteins that are differentially distributed in nociceptors. The opening and closing of voltage gated proteins are described as series of 'states' that represent specific protein shapes (conformations). These open and closed states undergo activation, inactivation and deactivation as they transition from closed to open to closed states. Activation and deactivation are voltage dependent, while inactivation, although influenced by voltage, occurs in the absence of voltage changes. In an attempt to understand the basis of shifts in neural excitability and the development of spontaneous activity, we examined the influence of permethrin on a subset of voltage gated proteins that are known to influence action potential duration.

Task 3: We will examine the acute effects of permethrin on Na_v proteins in skin, muscle and vascular nociceptors

While there have been limited studies on the influence of permethrin on neural protein function, the influence of structurally similar pyrethroid neurotoxicants has been studied extensively. The permethrin/pyrethroid promotion (or obstruction) of neural activity has been associated with changes in activation, steady-state inactivation and deactivation of insect Nav proteins (Narahashi et al., 1998; Narahashi, 2000; Ray and Fry, 2006; Soderlund, 2010). This array of functional modulation of Nav has been documented in mammalian neurons across a structurally and functionally diverse family of mammalian Nav (e.g., $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, $Na_v1.8$), but the specifics of the modulation differ both qualitatively and quantitatively (Song and Narahashi, 1996; Song et al., 1996a,b; Smith and Soderlund 1998; Motomura and Narahashi, 2001; Dekker, et al., 2005; Choi and Soderlund, 2006; Meacham, et al., 2008; Breckenridge et al., 2009; Tan and Soderlund, 2009). The relationship of $Na_v1.3$ to neural injury and differential distribution of

Nav proteins in the central ($Na_v1.2$) and peripheral nervous system ($Na_v1.6$, $Na_v1.8$) may be significant (Rush et al., 2007; Dib-Hajj et al., 2009).

In the studies below, we demonstrate that permethrin exhibits powerful influences on the voltage dependence of activation and deactivation of nociceptor $Na_v1.8$. As we observed with action potentials, these effects were manifested across tissue specific nociceptor classes that expressed $Na_v1.8$ (Jiang et al., 2011). Changes in deactivation can explain AP elongation that were seen across all tissue specific classes, but neither these nor activation shifts can explain the specific effects we observed on muscle nociceptor excitability and spontaneous activity that were limited to muscle nociceptors.

Muscle nociceptors, as well as many other nociceptor types, express the TTXr $Na_v1.8$ protein (Djouhri et al., 2003a,b; Amir et al., 2006; Jiang et al., 2011). Accordingly, we applied protocols to examine the activation, inactivation and deactivation of $Na_v1.8$ in the presence of permethrin. Activation reflects the tendency for $Na_v1.8$ protein to form action potentials. These data were assessed by formation of classic sigmoidal activation curves where a $V_{.50}$ activation statistic reflects the voltage at which half of the proteins are activated. A leftward, hyperpolarizing, shift in the activation $V_{.50}$ for $Na_v1.8$ (more negative) indicates a cell that is more likely to form action potentials that will be perceived as pain. Inactivation was also assessed by classic methods. A $V_{.50}$ was derived that reflected the voltage at which half of the $Na_v1.8$ proteins were available to be activated. A depolarizing (more positive) shift of the inactivation $V_{.50}$ indicates that a larger portion of the total pool of $Na_v1.8$ proteins are capable of being activated. Deactivation refers to the movement of proteins from an open to a closed state due to a hyperpolarizing ion flux. The slowing of deactivation leads to great excitability. Deactivation was assessed by deriving time constants (tau_{deact}) that characterized the exponential decay of $Na_v1.8$ mediated current in proportion to a series of deactivating voltage steps (see Appendix, p. 73). Recordings were obtained from skin, muscle and vascular nociceptors.

The tendency to form action potentials (excitability) is highly related to the threshold of Na_v protein activation. We examined whether the activation of $Na_v1.8$ was influenced by the acute application of permethrin (10 μM). Skin, muscle and vascular nociceptors were identified in the usual manner. A Na-Iso solution (Appendix, p. 74) was used to separate Na^+ currents from other voltage sensitive membrane currents. Permethrin was applied for 2 minutes. Subsequently, a

$V_{.50}$ was derived from sigmoidal functions fit to the normalized voltage-conductance (G) plots. Contrasts were then made between the $V_{.50}$ of ETOH/ETOH treated cases and ETOH/Permethrin treated cases. Consistent with increased excitability, permethrin shifted the midpoint of excitation ($V_{.50}$) in the hyperpolarizing direction (figure 21). That is, $Na_v1.8$ proteins exposed to permethrin had a far greater sensitivity to voltage changes and were much more likely to form action potentials. No changes in peak amplitude were observed (0 mV test; figure 22).

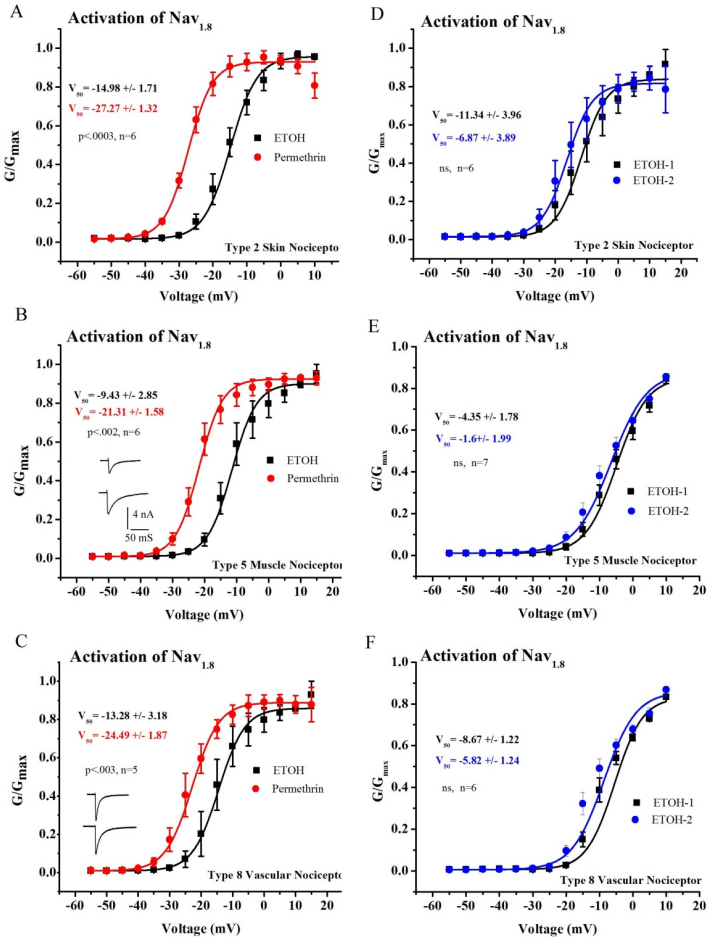

Figure 21. Permethrin Shifted the Voltage Dependent Activation of $Na_v1.8$. Depolarizing shifts in the activation $V_{.50}$ suggested greater excitability following permethrin exposure (10 μM): **A)** Skin nociceptors; **B)** Muscle nociceptors; Representative traces are presented as inserts. Upper trace: pre-permethrin; lower trace: post permethrin (0 mV activation step); **C)** Vascular nociceptors. Representative traces are presented as inserts. Upper trace: pre-permethrin; lower trace: post permethrin (0 mV activation step). Scale bars in 'B' apply. Control vehicle tests (ETOH-1 first exposure; ETOH-2 second exposure) did not produce any significant shifts of $V_{.5}$ (.001% ETOH; different cells from A, B, C): **D)** Skin nociceptors; **E)** Muscle nociceptors; **F)** Vascular nociceptors. Comparisons between the first ETOH-1 and 'Vehicle' tests (.001% ETOH) were non-significant.

Even though actual excitability was only increased in muscle nociceptors (figure 20B), permethrin affected skin, muscle and vascular nociceptors $V_{.5}$ in a similar manner and degree (figure 21). The $V_{.50}$ for activation shifted more than 10 mV in all nociceptor classes. Clearly nociceptors were highly vulnerable to the acute influence of permethrin.

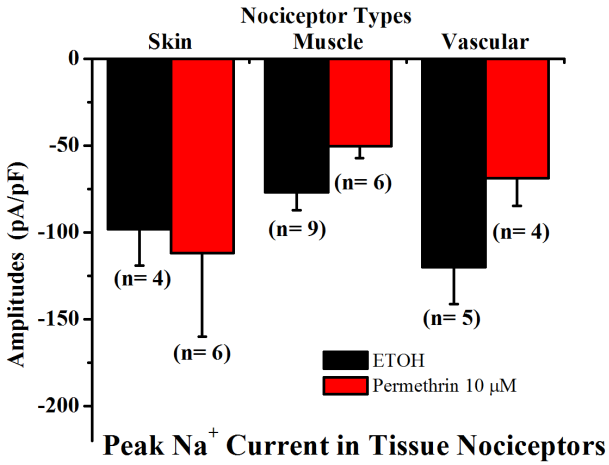

Figure 22. Acute Permethrin did not Increase the Amplitude of $Na_v1.8$. At the test voltage of 0 mV, the normalized peak amplitude of $Na_v1.8$ in tissue specific nociceptors remained relatively stable compared to ETOH treated controls.

In a cell body, action potentials are terminated by voltage dependent deactivation. Action potential prolongation that we observed (figure 19) could have been due to poor deactivation of $Na_v1.8$. We directly assessed the influence of permethrin on deactivation of nociceptor $Na_v1.8$. Deactivation time constants (tau_{deact}) were determined by fits of exponential decay functions to Na^+ currents that were terminated 8 msec after a strong depolarizing voltage step (see Appendix, p. 73). As previously demonstrated with other type I pyrethroids (Soderlund, 2010), exposure to permethrin induced very large deactivation tail currents (figure 23). Relative to control cases, all permethrin treated nociceptors exhibited significant increases in the deactivation time constants consistent with a prolongation of action potential duration (e.g., tau_{deact} = 11.3 +/-1.4 vs 5.9 +/- 0.47 ms at -60 mV; type 5 muscle, $p<.01$). The influence of permethrin became strongly voltage dependent over the test voltages, and reached a maximum change near the holding potential (i.e., -40 mV test, V_H= -60 mV, figure 23). In order to evaluate the voltage dependent changes, exponential functions were fit to individual cell, voltage-tau, records and a constant '*k*' was derived from this equation that characterized the growth of the function with voltage (see

Appendix, p. 74). Comparison of *k* between ETOH and permethrin treated skin, muscle and vascular nociceptors were highly significant (p<.002, .008 and.02; figure 23 imbeded).

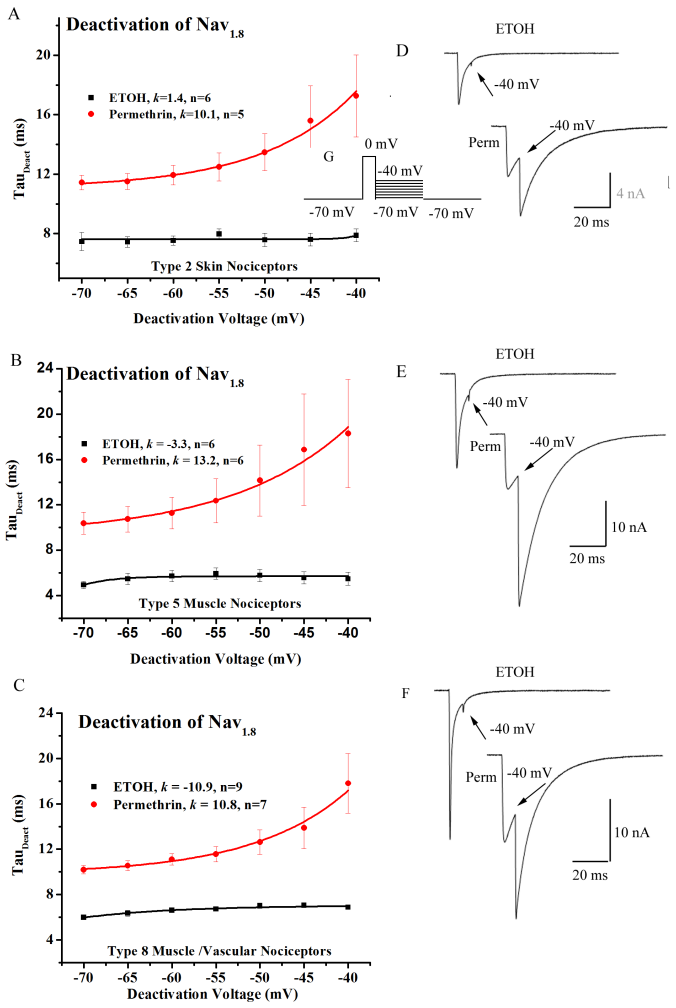

Figure 23. Permethrin Shifts Voltage Dependence of Deactivation in All Tissue Specific Nociceptors. A-C) ETOH exposed cells exhibit a relatively consistent pattern of deactivation across membrane voltages (black curves). Following treatment with permethrin, all deactivation time constants were increased and a strong voltage dependence appeared (red curves). There were no statistical differences amongst tissue specific nociceptors. Values of *k* are shown imbedded. **D-F)** Representative traces illustrate the deactivation tail current associated with permethrin and ETOH treated neurons. All traces taken from a single deactivation voltage step to -40 mV. The arrow signifies the point at which the voltage is abruptly clamped at -40 mV. **G)** A schematic of the deactivation protocol. The voltage step to 0 mV was 8 ms long. The deactivation voltage steps were 150 ms. 10 µMm permethrin applied for 2 minutes.

Nav proteins are highly expressed in cells and axons, but may not be 'available' for forming action potentials. Following activation, Na_v proteins will enter an inactivated state from which they can be released (made available) by hyperpolarizing (very negative) potentials. Inactivation

also reflects the tendency for Nav proteins to close from the open (activated) state to a closed (inactivate) state despite the presence of activating voltage. It is contrasted by deactivation which reflects the tendency to close from the open state when the voltage shifts to one at which the protein is normally closed. This feature is classically studied by holding Na_v proteins at strongly activating pre-pulse voltage (steady state) and then gradually releasing the inactivation that forms by systematically stepping the conditioning pre-pulse to hyperpolarizing voltages. A $V_{.50}$ for inactivation can be determined from Boltzman functions fits to Na_v currents (Appendix, p. 76). After performing tests of steady state inactivation in isolated Na^+ currents, we were unable to demonstrate any influence of permethrin on the voltage dependence of steady state inactivation of $Na_v1.8$ in tissue specific nociceptors (figure 24). The tau_{inact} was greatly increased (figure 24 inserts).

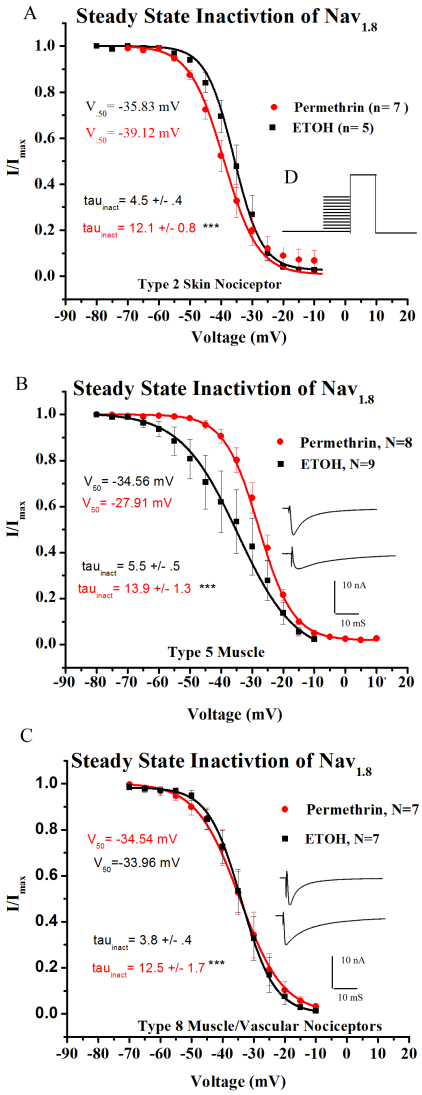

Figure 24. Permethrin and Fast Inactivation of $Nav_{1.8}$ A-C) Voltage dependent inactivation of $Nav_{1.8}$ was not significantly modified by application of 10 μM permethrin (2 minutes); The tau_{inact} (mS) was significantly increased in all cases. **D)** A schematic of the inactivation protocol. Inserts show representative traces (upper: ETOH; lower: Permethrin). Note the slowing of inactivation after permethrin. The cells were pre-pulsed in 5 mV steps from -10 to -80 mV. The test pulse was 0 mV, 150 ms.

The Influence of Permethin at Physiological Temperatures. Humans are highly resistant to pyrethroid poisoning. A number of factors (absorption, elimination, body temperature, innate sensitivity) contribute to the lower lethality of pyrethroids in humans (Ray and Frey, 2006; Narahashi et al., 2007). Pyrethroids are effective as insecticides due to their actions on insect Na_v proteins (Raymond-Delpech et al., 2005; Narahashi, 2000; Soderlund, 2010). However, mammalian Na_v proteins are structurally diverse and anatomically specialized to a much greater extent than those expressed in invertebrates. Although the functional significance is obscure, the influence of pyrethroids on distinct mammalian Na_v differ qualitatively and quantitatively (Ruigt, et al. 1987; Song et al., 1996a,b; Choi and Soderlund, 2006; Ray and Fry, 2006; Meacham, et al.,

2008; Breckenridge et al., 2009; Tan and Soderlund 2009, Soderlund, 2010). Much significance has been attached to thermal modulation of Na_v vulnerability. As the physiological temperature approaches 37° C, the actions of pyrethroids on the mammalian central nervous system (Song and Narahashi,1996; Motomura and Narahashi, 2000) diminished or abrogated (see also: Wang et al., 1972; van den Bercken et al., 1977; Cutkomp and Subramanyam, 1986; Salgado et al., 1989). This temperature dependence would seem to preclude pyrethroid actions on mammals whose body temperature hovers near 37° C. Nevertheless, it is well documented that exposure to permethrin formulations causes pain and paresthesia in humans lasting minutes to hours (Kolmodin-Hedman et al., 1982; Tucker and Flannigan, 1983; Flannigan and Tucker, 1985; Gotoh, et al., 1998; see Wolansky and Harrill, 2008). These conflicting pools of evidence are unresolved, but could indicate a broader spectrum of pyrethroid interaction with nervous system proteins contributing to spontaneous activity than previously thought. Accordingly, we examined the influence of temperature on: 1) permethrin initiated spontaneous activity; 2) non-Na_v proteins that contribute to spontaneous activity in nociceptors; and 3) Na_v protein physiology.

Studies were conducted on skin, muscle and vascular nociceptors. Following isolation of type 2, type 5 or type 8 nociceptors, the superfusion temperature was increased from 21 to 35° C. In molecular studies, Na_v currents were isolated and activation or deactivation protocols were applied in the same manner as the experiments above. Molecular studies were only conducted on type 2 nociceptors. In cellular studies, action potentials features were tested with the resting membrane potential corrected to -60 mV. After preliminary characterization in an ETOH containing solution, permethrin was applied continuously for 10 minutes while AP features and spontaneous activity was assessed. Additional studies were performed in which permethrin induced spontaneous activity was first established at 21° C before the superfusion temperature was changed to 35° C.

Permethrin Induced Shifts In $Na_v1.8$ Persist at Mammalian Physiological Temperatures. Studies were conducted on skin nociceptor $Na_v1.8$. Superfusion temperature was increased to 35° C and the deactivation protocol was applied in the presence of ETOH (.001%) or permethrin (10 μM; 2 min). Following tests at physiological temperature, the superfusion solution temperature was reduced to 21° C (ETOH). We observed that the influence of permethrin was greatly reduced at

35° C; however, a statistically significant slowing of deactivation clearly remained (figure 25). When superfusion temperatures were shifted to 21° C, the expected pronounced permethrin effects were readily visible. The substantial improvement of $Na_v1.8$ deactivation at 35° C would greatly contribute to the partial restoration of action potential duration. However, as a complete reversal did not occur, it is clear that a persistent failure to fully achieve normal $Na_v1.8$ deactivation could be a consequence of human exposure to permethrin, and, following prolonged exposure might lead to persistent CNS and/or PNS maladaptations (Binns et al., 2008).

At room temperature, permethrin greatly shifted the $V_{1/2}$ for $Na_v1.8$ activation in all nociceptor populations. Certainly this may have contributed to the development of spontaneous activity. For tests at elevated temperatures, skin nociceptors were isolated and experiments were conducted as previously described. As we observed with other cellular and molecular measures, temperature greatly reduced the influence of permethrin ($p<.005$; 21° vs 35° C), but significant shifts in the $V_{1/2}$ for activation were retained at 35° C ($p<.0002$; figure 25). As a result, an increased propensity to discharge would be expected in mammalian neurons chronically exposed to permethrin.

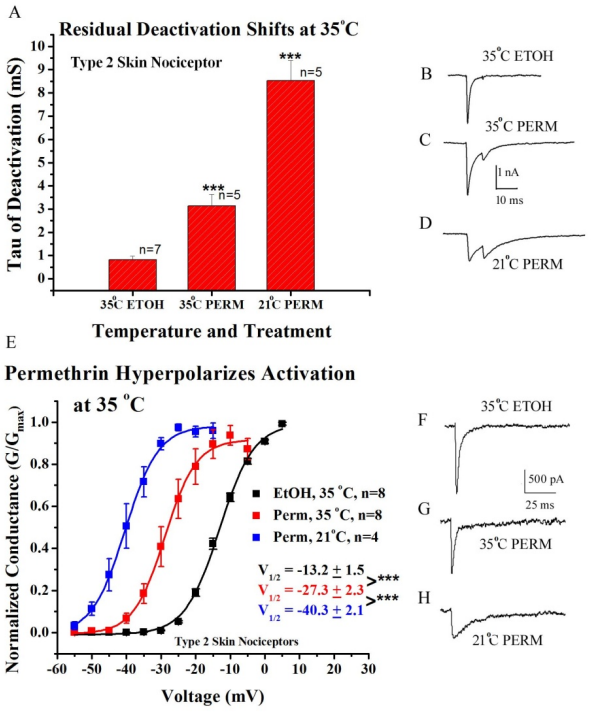

Figure 25 Physiological Temperature do not Fully Reverse the Influence of Permethrin on $Na_v1.8$. A) At 35° C the tau_{deact} was significantly increased by permethrin. Increases were much less than those previously observed at 21° C. When temperature was shifted to 21° C, the influence of permethrin was greatly enhanced. **B, C and D)** Representative traces of deactivation of $Na_v1.8$. **E)** At 35° C, the $V_{1/2}$ was significantly shifted in the hyperpolarizing direction following permethrin application. When the superfusion temperature was shifted to 21° C the shift was increased further. F, G and H) Representative traces at relevant temperatures.

Cellular Studies at Physiological Temperatures.

When permethrin was applied at physiological temperatures, muscle nociceptors exhibited far less spontaneous activity than was observed at room temperature conditions (figure 26D; van den Bercken et al., 1977; see also Motomura and Narahashi, 2000). Similarly, when spontaneous activity was initiated at 21° C and superfusion temperatures were subsequently increased to 35° C, action potential activity was greatly reduced or ceased altogether. In addition, the impressive permethrin-induced prolongation of action potential duration and abrogation of AHP were mitigated at mammalian physiological temperatures (figure 20A). Although AP duration was greatly reduced at 35° C, the permethrin treated neurons retained a significantly longer AP duration than the ETOH treated group. The associated obliteration of the afterhyperpolarization, documented in figure 19, was also largely reversed at 35° C in muscle nociceptors (figure 26).

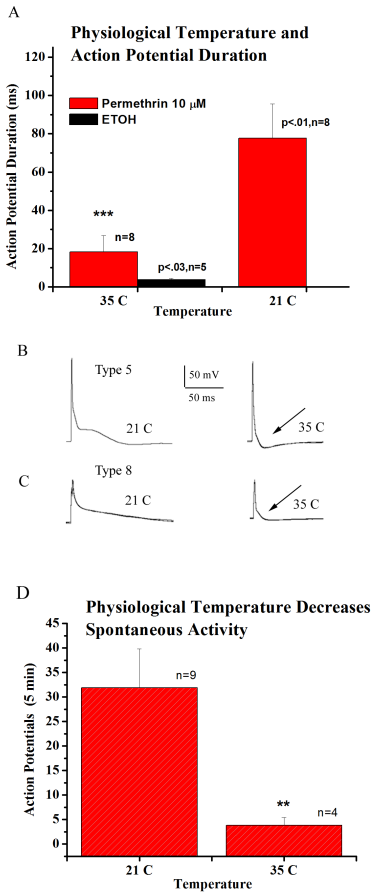

Figure 26. Thermal Modulation of Permethrin Influences on Action Potentials. A) Permethrin-induced increases in action potential duration was diminished at 35.5 C. Action potential duration remained significantly increased relative to ETOH treated neurons (type 5 and type 8 pooled). **B)** Representative action potentials of a type 5 muscle nociceptor at 21 and 35 C. Note the reappearance of an afterhyperpolarization period at 35 C (arrow). Three of 4 afterhyperpolarizations recovered in muscle nociceptors **C)** Representative action potentials of a type 8 nociceptor at 21 and 35 C. Note that the afterhyperpolarization did not recover in the two cases examined (arrow). **D)** Physiological temperatures significantly reduced the spontaneous action potentials evoked by permethrin (5 min observation; vascular nociceptors).

Acute Influence of Permethrin on K_v7 Mediated Currents

K_v7 refers to a family of voltage dependent proteins that are activated near the resting membrane potential. The flow of ions through K_v7 proteins oppose Na_v dependent activation while other K^+ channels, such as I_a, influence Na_v deactivation. As a result, K_v7 activation has the unique capacity to prevent the initiation of action potentials. A decline in K_v7 activity could have a substantial influence on neuronal excitability and spontaneous discharge (Brown and Selyanko, 1985; Wang and McKinnon, 1995; Marrion, 1997; Robbins, 2001; Brown and Passmore, 2009). Portions of the K_v7 protein family have been indentified in sensory neurons (Passmore et al., 2003; Linley et al., 2008). Although the lethal influence of pyrethroids are manifested via Na_v proteins, it is known that pyrethroids can also modulate function of a number of other neuronal ion channel proteins (Ray and Fry, 2006). In order to understand how permethrin initiated spontaneous activity in muscle nociceptors, we examined whether K_v7 proteins were influenced by the acute application of permethrin.

Task 4: We will assess nociceptors for changes in K_v7 function that could contribute to chronic and widespread pain.

Skin, muscle and vascular nociceptors were isolated as above. The K-iso solution containing .001% ETOH was applied during presentation of a protocol designed to isolate deactivating K_v7 currents (see figure 27A imbed). Subsequently, ETOH or permethrin was applied for 2 min, followed by the K_v7 specific inhibitor linopirdine or linopirdine+permethrin. Linopirdine sensitive K_v7 currents were identified by subtraction. We were unable to identify any influence of permethrin on K_v7 amplitude or voltage dependence in muscle or vascular nociceptors (figure 27). Type 2 nociceptors did not exhibit any linopirdine sensitive currents and therefore were not subjected to permethrin testing.

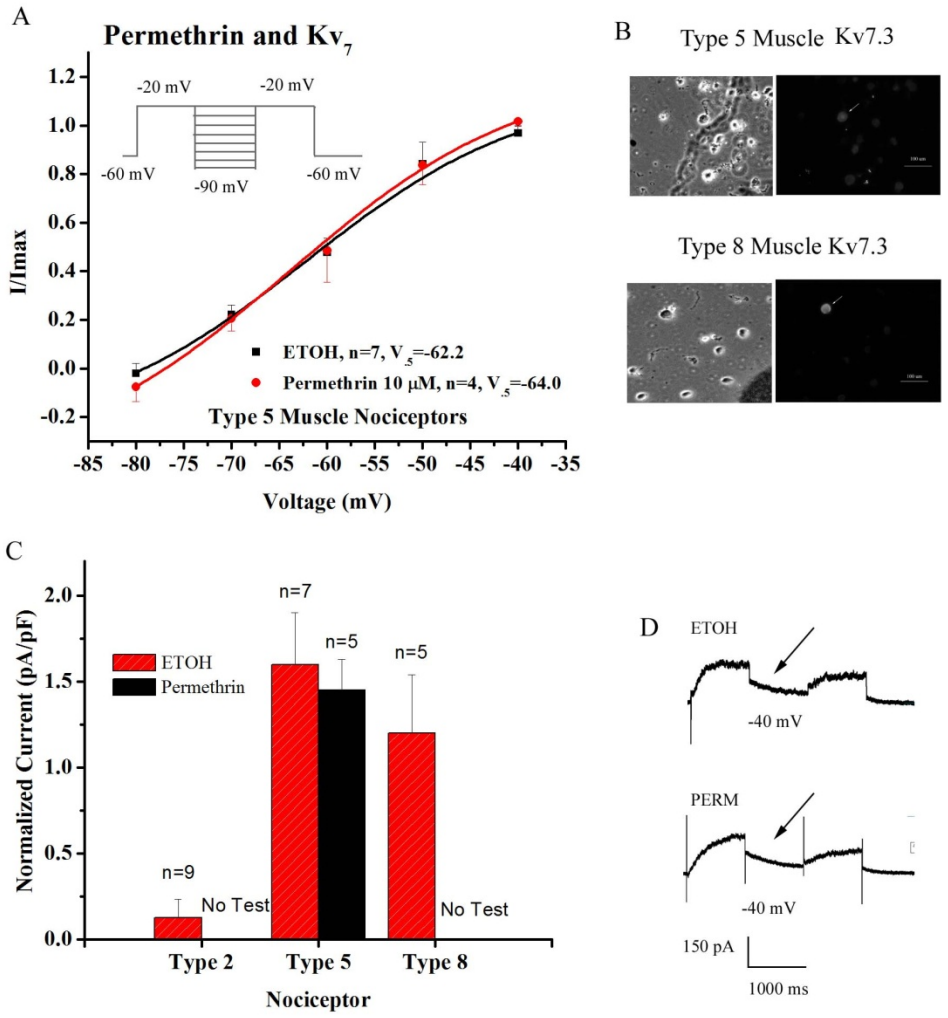

Figure 27. Permethrin did not Modify Currents Passing Through K_v7 Proteins. A) Voltage dependence of K_v7 is similar after 4 minute permethrin treatment (type 5 muscle nociceptors). **Insert:** A schematic representation of the voltage protocol used to evoke K_v7 currents. **B)** Type 5 and type 8 cells stain positively for Kv7.3 protein. **C)** Summary data of the averaged peak amplitude of permethrin and ETOH treated K_v7 proteins expressed in type 2 (skin), type 5 (muscle) and type 8 (vascular) nociceptors. Skin nociceptors did not express a linopirdine sensitive current. **D)** Representative traces of linopirdine sensitive current (subtraction currents in the presence of ETOH or permethrin) evoked at -40 mV. The arrows indicate the linopirdine sensitive 'tail' currents.

Key Research Accomplishments:

*Additions to the list of accomplishments that were achieved in this reporting period are shown in *italicized print*

Chronic Exposure to Permethrin, Chlorpyrifos and Pyridostigmine Bromide:

- Persistent voltage and amplitude changes in K_v7 protein currents specifically in vascular nociceptors
- Persistent amplitude changes in K_{DR} protein currents specifically in vascular nociceptors
- Persistent increases in membrane resistance specifically in vascular nociceptors
- Muscle nociceptors unaffected ($Na_v1.8$, K_v7, K_{DR})
- **Persistent amplitude and kinetic changes in vascular and muscle nociceptor $Na_v1.9$*
- *Heightened spontaneous discharge in vascular nociceptors*
- *Increased action potential duration and afterhyperpolarization*
- Skin, vascular and muscle nociceptor $Na_v1.8$ protein inactivation and deactivation unaffected
- Chronic effects of neurotoxicants/PB differ from acute effects of permethrin

Acute effects of permethrin on the pain system:

- Permethrin activates muscle nociceptors but not skin or vascular nociceptors via $Na_v1.8$
- Permethrin increases muscle nociceptor excitability, but not the excitability of skin or vascular nociceptors
- Permethrin increases skin, muscle and vascular nociceptor AP duration
- Permethrin accentuates skin, muscle and vascular nociceptor voltage dependent activation of $Na_v1.8$ protein (hyperpolarized $V_{.50}$)
- Permethrin retards skin, muscle and vascular nociceptor voltage dependent deactivation of $Na_v1.8$ protein
- Permethrin slows the rate (decay) of $Na_v1.8$ inactivation of muscle, skin or vascular nociceptors
- Permethrin has no influence on the normalized peak amplitude of skin, muscle or vascular nociceptor $Na_v1.8$
- Permethrin has no influence on voltage dependence of skin, muscle or vascular nociceptor inactivation of $Na_v1.8$
- Permethrin has no acute influence on K_v7 amplitude or activation in muscle or vascular nociceptors
- *At physiological temperatures (~35° C) the acute influence of permethrin on $Na_v1.8$ activation and deactivation are greatly reduced but not eliminated*

- At physiological temperatures (~35° C) the acute influence of permethrin on spontaneous activity and action potential duration and afterhyperpolarization are greatly reduced but not eliminated.

Reportable Outcomes

Abstracts Presented:

Jiang, N., Nutter T. J. and Cooper, B.Y. The Insecticide Permethrin Activates Muscle but not Skin Nociceptors. Presentation at the International Association for the Study of Pain, August, 2012.

Cooper, B.Y., Nutter, T. and Jiang, N. Persistent K^+ channel dysfunction after chronic exposure to insecticides and pyridostigmine bromide. Presentation at the Annual Meeting of the Society for Neuroscience, November 2012.

Publications:

Jiang N, Nutter TJ, Cooper BY. Molecular and cellular influences of permethrin on mammalian nociceptors at physiological temperatures. Neurotoxicology. 2013. 37:207-19.

Manuscripts Submitted:

Nutter T., Jiang, N., and Cooper, B.Y. Persistent Na^+ and K^+ Channel Dysfunctions after Chronic Exposure to Insecticides and Pyridostigmine Bromide. Submitted to Neurotoxicology

Manuscripts in Preparation:

Nutter T. and Cooper, B.Y. Persistent Changes in the Physiology of $Na_v1.9$ Following Chronic Exposure to Insecticides and Pyridostigmine Bromide.

Abstracts Submitted:

Cooper, B.Y. and Nutter, T.J. Persistent modification of $Na_v1.9$ following chronic exposure to insecticides and pyridostigmine bromide. Accepted for Presentation at the Annual Meeting of the Society for Neuroscience, November 2013.

Funding applied for based on work supported by this award

We have applied for funding to the PRMRP (2013 White Paper) to develop nanomedicine devices to treat a variety of pain syndromes associated with military service. It is noted in the application to include applications for treating GWI.

Laser Activated Nociceptor Directed Nanomedicine Device for Service Related Chronic Pain, PR130176

Research opportunities received based on experience/training supported by this award

We received an award to further study molecular and cellular events associated with GWI pain:

Persistent Neural Membrane Protein Misregulation Following Neurotoxicant Exposure

GW120039, funded, start date: Aug 1, 2013.

Conclusions

We examined whether chronic exposure to combinations of two neurotoxicants (chlorpyrifos and permethrin) with pyridostigmine bromide could produce a delayed chronic pain condition in rats; and whether corresponding molecular changes would occur in neurons coding for pain in skin, muscle or vasculature. Following a 30-60 day exposure to neurotoxicants/PB we observed increased pain behavior only during periods that coincided with neurotoxicant dosing. Pain indices faded after dosing ceased. Possibly the behavioral measures we chose cannot assess the sort of pain induced by NTPB.

We have reported a number of molecular changes in nociceptor physiology after exposure to NTPB that could result in a chronic pain condition. Some of these, taken in isolation, would be considered essentially excitatory ($Na_v1.9$ conductance; $Na_v1.8$ decay) or essentially inhibitory in nature (K_v7 conductance; tau_{act} of $Na_v1.9$; tau_{decay} of $Na_v1.9$). In other instances we have observed correlates of protein channel changes (membrane resistance) but have not yet identified a specific molecular event that would account for the observation. Some of the changes in molecular physiology are more persistent than others, but all extend beyond the period of direct treatment with NTPB and persist at least 4 to 8 weeks after the exposure ended. Some are seen to extend up to 12 weeks (tau_{act}, $Na_v1.9$). In the author's judgment, the changes in $Na_v1.9$ and K_v7 conductance are the most important molecular adaptations to NTPB exposure. In any case, many of the molecular adaptations tend to oppose the action of the other, and it is likely that the inhibitory changes evolved to oppose the excitatory changes in an attempt to maintain normal excitability of nociceptive neurons. It is difficult to say at the molecular level, which effect would dominate the other, such that the net effect would be greater or lesser nociceptor excitability. Normal adaptive mechanisms would attempt to keep these in a neutral balance.

The functional changes revealed in the cellular studies, permit us to assess the net effect of increased $Na_v1.9$ conductance and how it plays out against the increase in K_v7 conductance. When interpreting the net effect, which could clearly include maladaptations of other molecular species not yet examined, the cellular changes provide the best source of insight. In the cellular studies we have documented an increase in AP duration, AP afterhyperpolarization, AP discharge at 35° C and linopirdine-released discharge.

It is possible that the changes in AP duration, as a consequence of NTPB exposure, could have played an important role in initiating and perpetuating Ca^{++} dependent neuroplastic changes leading to increased spontaneous activity and ultimately a chronic pain condition. Such changes would have been significant both during exposure, when permethrin directly prolongs AP duration via direct actions on $Na_v1.8$, as well as after treatment, when shifts in $Na_v1.9$ conductance could have continuing influences on AP duration.

One of the purposes of AP duration is to limit the amount of Ca^{++} entering the cell during an action potential. Because voltage activated Ca^{++} channels are activated during the action potential, a lengthening of the action potential duration would permit more Ca^{++} to enter the neuron. Our observation of significant increases in AP afterhyperpolarization duration in NTPB treated rats supports the possibility that more Ca^{++} was entering the cell; because, among other things, Ca^{++} would activate the Ca^{++} activated K^+ channels that produce the afterhyperpolarization. The broadening of nociceptor action potentials could initiate a wide range of Ca^{++} dependent intracellular events, including: 1) increased neurotransmitter release; 2) activation of Ca^{++} dependent protein kinases, and phospholipases that are normally associated with inflammation; and 3) gene transcription (Park and Luo, 2010). Over a period of months of exposure, the consequences of these perturbations might result in significant molecular maladaptations to Na_v or other membrane proteins expressed in the affected afferent neuron pool and their post-synaptic targets in the spinal cord. Because $Na_v1.9$ would necessarily be activated with every AP, the increase in AP duration could have been due to the increased conductance of this channel protein. And because of its potential contribution to amplifying sub-threshold depolarizing ion fluxes, $Na_v1.9$ could supply the driving force for spontaneous discharge that we have documented in vascular, and to a lesser extent muscle nociceptors.

The changes in $Na_v1.9$ physiology may have derived from the direct actions of permethrin, a substance known to interact strongly with Na_v. Although much study has been directed at the relationship between pyrethroids and Na_v, (Narahashi et al., 1998; Narahashi, 2000; Ray and Fry, 2006; Soderlund, 2010);) the actions of pyrethroids on $Na_v1.9$ have never been documented. This is likely due to the fact that $Na_v1.9$ has only been recently discovered, it is only expressed in nociceptors, and the interaction of Na_v with pyrethroids has been largely been considered a completed field of study (Tate et al., 1998; Cummins et al., 1999; Fang et al., 2002). We are

currently investigating the acute influences of permethrin on $Na_v1.9$ as part of an EWOF to complete Task 2 of the SOW. These results will be presented in the final report (due January 14, 2014).

From this standpoint we can interpret the increase in K_v7 as an attempt of intra-neuronal regulatory processes to suppress excessive nociceptor excitability induced by the direct Na_v promoting influence of permethrin during the exposure period, and as a continuing attempt in the post exposure period to oppose residual excessive driving force of a maladapted $Na_v1.9$. Our studies in which vascular nociceptor spontaneous activity was 'released' by linopirdine, a specific inhibitor of K_v7, promotes this interpretation. Namely, the increased capacity of $Na_v1.9$ to amplify sub-threshold depolarizations has led to an increased K_v7 conductance in order to keep these sub-threshold fluxes in check. When K_v7 is inhibited, by linopirdine in our experiments, the increased depolarizing currents are released, and spontaneous discharge is observed. Of course, spontaneous discharge in nociceptors codes for pain. Other interpretations are also worthy of consideration. Namely, that the changes in K_v7 may not be reactive to chronic permethrin-induced changes excitability, but could represent direct residual modulation by other neurotoxicants that were present in the Gulf theatre.

K_v7 channel proteins play an important role in neuronal spontaneous discharge (Marrion, 1997; Brown and Passmore, 2009). K_v7 channels were historically identified as the source of 'M-currents' due to their regulation by muscarinic acetylcholine receptors. Disturbances in cholinergic physiology would not be surprising in veterans of the GW. Persian Gulf soldiers were exposed to multiple anticholinesterases during their deployment. Exposures derived, not only as self-applied insecticides (i.e., chlorpyrifos) and nerve gas prophylactics (PB) but also from the intentional and/or accidental release of nerve agents (Sarin) and other carbamates or organophosphates with which they had voluntary and/or involuntary contact (Fricker et al., 2000; Haley et al., 2012; see Binns et al., 2008). Chronic exposure to varied cholinesterase inhibitors might alter the physiology of cholinergic receptors and their downstream targets (K_v7). Therefore, a pathway can be seen from perturbations of $Na_v1.9$ (permethrin influences) and inhibition of K_v7 (chlorpyrifos, PB influences) to spontaneous discharge in nociceptor populations and chronic pain. Although not a part of this SOW, one implication of this hypothetical scenario, is a heightened sensitivity of vascular nociceptors to acetylcholine.

Acetylcholine functions both as a neurotransmitter for skeletal muscle and also as a pro-inflammatory mediator released from a variety of non-neuronal cells (Cooper and Rau, 2005). The inhibition of K_v7 by acetylcholine is the in vivo correlate of exposure to linopirdine in vitro, and could be a candidate instigating factor in the chronic pain of GWI.

A less recognized influence of cholinesterase inhibitors, including PB and chlorpyrifos, is the increased expression of M2 muscarinic receptors (Abou-Donia et al., 2002; Abou-Donia et al., 2003; Abou-Donia et al., 2004; but see Abu-Qare and Abou-Donia, 2003; Abdel-Rahman, et al 2004). There is also evidence of direct binding of the chlorpyrifos metabolite, chlorpyrifos-oxon, to muscarinic receptors (M2, M4; Huff and Abou-Donia, 1994; Huff et al., 1994; Howard and Pope, 2002). These muscarinic receptors are present in rat dorsal root ganglion (Haberberger et al., 1999; 2000). Although we do not know about the specific distribution of M2 receptors in tissue specific nociceptors, we have observed that both type 5 and type 8 muscle and vascular nociceptors can be activated by muscarinic agonists (Rau et al., 2013). Accordingly, in addition to being nociceptor populations that are demonstrated to respond to NTPB with maladapted K_v7 and $Na_v1.9$, they are also candidates for chronic misregulation of muscarinic receptors by anticholinesterases. That vascular nociceptors are the focus for many of the molecular and functional defects associated with NTPB might be reflective of known cholinergic vascular reflex dysfunctions in veterans suffering from GWI (Haley et al., 2009; Liu et al., 2010; Li et al., 2011).

Despite the interesting functional changes we observed in Na_v and K_v channels, the lack of a measureable behavioral correlate remains troubling. It is difficult to definitively tie the molecular and cellular changes in nociceptors to a chronic pain condition in the absence of a behavioral correlate. The lack of this behavioral correlate has prevented us from executing Task 5. Task 5 called for the use of agents to reverse the pain produced by the neurotoxicants/PB. As we could not measure any pain, the Task could not be performed. Perhaps the behavior measures we have chosen cannot measure the type of pain associated with NTPB. A vascular or ischemic type pain is not directly measureable by known behavioral tests. It is also possible that the molecular changes are 'pre clinical'. Therefore, pain does not accompany these molecular changes because a component that aggravates or exposes the molecular defects is absent from the NTPB exposure regimen. This component could be an additional neurotoxicant

that was present in the Gulf (Binns et al., 2008). The component could also be insufficient dosing of the present set of neurotoxicants.

Using a separate source of funding and different animal protocol we have found evidence that the addition of DEET to the mix of neurotoxicants does produce lasting pain in NTPB exposed rats. DEET was one of the many insecticides and repellants that veterans were exposed to during their deployment in the Persian Gulf (Fricker et al., 2000; Binns et al., 2008). We hope to utilize this new treatment protocol in future studies with the approval of the CDMRP/ACURO.

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Appendix

Statement of Work

Task 1: We will examine the development, time course and persistence of a myalgia in rats exposed to combinations of neurotoxicants pyridostigmine bromide, permethrin and chlorpyrifos.

Time Frame: 10 months (4-14)

Young adult male rats (n=144-450) will be systematically treated with 3 neurotoxicants for periods of 30-60 days. On a weekly basis, we will test for pressure myalgia and general malaise (open field activity). Testing will continue for 3 months after termination of neuotoxicant dosing. Up to 450 rats will go through the dosing and behavioral testing that terminates at various stages with harvesting of DRG cells for electrophysiological studies. Only 144 rats are required for the behavioral studies.

Task 2: We will assess nociceptors for changes in Na_v function that could contribute to chronic and widespread pain following treatment with 3 neurotoxicants

Time Frame: 14 months (8-22)

Rats (n=225) are harvested from behavioral studies assessing myalgia following treatment with 3 neurotoxicants. Rats with and without behavioral changes are sacrificed at 1, 2 and 3 months following the 30-60 treatment regimen. For studies involving Na_v proteins, we will examine muscle, vascular and skin nociceptors that express $Na_v1.8$ and $Na_v1.9$. We will harvest DRG containing nociceptor cell bodies of skin, muscle and vascular nociceptors identified in DiI tracing studies and assess these for shifts in the physiology (voltage dependent activation and inactivation; kinetics of evoked and tail currents) of $Na_v1.8$ and $Na_v1.9$. Complementary current clamp studies assess action potential excitability. Delay and chronicity is assessed over a 3 month post-treatment period. Comparisons are made between behavioral tests and the time course of nociceptor malfunction. Acute effects are contrasted with chronic influences.

Task 2: We will assess nociceptors for changes in Na_v function that could contribute to chronic and widespread pain following treatment with 3 neurotoxicants

Task 2a: Assess Na_v Physiology in Muscle Nociceptors

Task 2b: Assess Na_v Physiology in Vascular Nociceptors

Task 2c: Assess Excitability in Muscle Nociceptors

Task 2d: Assess Excitability in Vascular Nociceptors

Task 2e: Assess Excitability in Skin Nociceptors

Task 2f: Assess Na_v Physiology in Skin Nociceptors:

Task 3: We will examine the acute effects of permethrin on Na_v proteins in skin, muscle and vascular nociceptors

Time Frame: 4 months (4-8)

Rats are used as experimental subjects (n=40). Although permethrin has demonstrated acute influence on Na_v in expression systems, and other type 1 pyrethroids are effective in DRG sensory neurons, no one has examined the influence of permethrin on specific nociceptor populations (muscle, skin, vascular). In these neurons the structural phenotype of $Na_v1.8$ may be the same, yet the pathways that regulate excitability and expression may be distinct. Moreover, the effect of permethrin on $Na_v1.9$ has never been examined.

Task 3a: Assess the Acute Influence of Permethrin on Na_v Physiology in Muscle Nociceptors

Task 3b: Assess the Acute Influence of Permethrin on Na_v Physiology in Vascular Nociceptors

Task 3c: Assess the Acute Influence of Permethrin on Na_v Physiology in Skin Nociceptors

Task 4: We will assess nociceptors for changes in K_v7 function that could contribute to chronic and widespread pain.

Time Frame: 16 months (6-22)

Muscle and vascular nociceptors express a K_v7-like current that is sensitive to Oxo-M and linopirdine. We will use optical recording methods to assess spontaneous activity in nociceptors.

We will use whole cell patch methods to identify the type of nociceptor exhibiting spontaneous activity and determine the status of K_v7 in these nociceptors. We will use agents that activate/suppress K_v7 (e.g., retigibine; XE911; linopirdine) and down stream pathways (PIP_2, $IP_3/Ca^{++)}$ to assess the contribution of this voltage sensitive protein to the spontaneous activity. Chronicity is assessed over a 3 month post-treatment period. DRG are harvested from PB and permethrin and chlorpyrifos treated rats (n=225) exhibiting myalgic symptoms as determined in Task 1. Control groups receive vehicle pretreatments or single agents over the same time course.

Task 4a: Assess K_v7 Physiology in Muscle Nociceptors

Task 4b: Assess K_v7 Physiology in Vascular Nociceptors

Task 4c: Assess Spontaneous Activity in Nociceptors

Task 4d: Assess the Nociceptor Phenotype Exhibiting Spontaneous Activity

Task 5: We will assess the capacity of pharmaceutical agents to reverse a neurotoxicant dependent myalgia.

Time Frame: 6 months (18-24)

After identification of an effective dose to produce a persistent myalgia and the molecular dysfunction(s) that parallel this myalgia (Tasks 1, 2 and 4), we will treat groups of rats (n=48) with the specific dosing procedure required. Following manifestation of the delayed behavioral change, we will begin a treatment series using agents targeting those proteins that were identified in Tasks 2 and 4. Behavior scores will be tracked during the treatment period to assess recovery. If rats return to normal levels, treatment dosing will discontinue after 3 weeks. Behavioral studies will continue after treatment to determine if behavior scores remain stable post-treatment

Methods

Acute Studies of Permethrin

Preparation of Cells. Young adult male Sprague-Dawley rats (90-110 g) were anesthetized (Isoflurane) and euthanized by decapitation. The dorsal root ganglia were excised, digested in a solution containing type 1 collagenase and Dispase II. The procedure has been described in detail previously (Petruska et al., 2002). Isolated neurons were plated on 8-10, 35 mm Petri dishes. Neurons were bathed continuously in Tyrode's solution, containing (in mM) 140 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. Electrodes (2-5 MΩ) were formed from boroscilate glass stock that was pulled to a suitable tip size by a Sutter P1000 (Sutter Instruments, Novato, CA). Recordings were completed within 2-10 h after plating. Only one cell was used per Petri dish. Studies were conducted at room temperature unless otherwise noted. All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and Acuro.

Recording and Characterization of Skin, Muscle and Vascular Nociceptors. For electrical recordings, conventional patch techniques were used to achieve the whole cell mode. Whole cell recordings were made with an Axopatch 200B or 700 (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and a Digidata 1322A (Molecular Devices). Series resistance (R_s) was compensated 65-70% with Axopatch compensation circuitry. Whole cell resistance and capacitance was determined by the Clampex software utility from a capacitive trace. Recorded currents were sampled at 10-20 kHz and filtered at 2 kHz (Bessel filter). Borosilicate pipettes were used (Sutter Instruments; uncoated) and pulled to a tip resistance of 2-4 MΩ.

Cells were classified as skin, muscle or vascular nociceptors according to the pattern of voltage activated currents evoked by three protocols (figure A1; Petruska et al., 2002; Rau et al., 2007). All reported data was derived from recordings made on type 2 (C nociceptor), type 5 (C or type IV nociceptor), and type 8 (Aδ or type IV nociceptor). Type 2 and type 5 were identified as skin and muscle nociceptors, respectively in prior tracing studies (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2012). Type 8 nociceptors were traced form all tissue sites (skin, muscle, colon,

mucosa; Rau et al., 2007; Rau et al., 2012) and are presumed to be vascular in origin. Consistent with their vascular designation, type 8 nociceptors co-express SP and CGRP (Petruska et al., 2002; Edvinsson et al., 1990; Jansen et al., 1992). We refer to these as 'putative' vascular nociceptors because we have yet to positively confirm their vascular innervation. It is possible this class innervates other widely spread tissues in addition to, or to the exclusion of, blood vessels (e.g., fascia, nerve sheath). Although the nociceptors we have tested in these studies are important classes of capsaicin (heat) sensitive nociceptors, additional classes of nociceptors can be traced from muscle and skin (Rau et al., 2012).

Classification of nociceptors into 'types' was performed by presentation of a series of depolarizing or hyperpolarizing characterization protocols as described previously in detail (Petruska et al., 2002). The technique is based upon original studies by Scroggs that were subsequently extended to describe more than 17 classes (Cardenas et al., 1995; Rau et al., 2012). Briefly, nociceptors differentially express multiple forms of voltage activated Na^+ (Na_v), hyperpolarization activated (I_H) and 'A-type' K^+ currents that can be distinguished by their voltage dependence, threshold, amplitude and kinetics. The identification of these currents by depolarizing or hyperpolarizing protocols permits classification of neurons into uniform subclasses on electrophysiological criteria alone. Subclassified nociceptors have been shown to exhibit distinct and internally uniform expression of serotonergic, cholinergic, purinergic, opiate, ASIC, TRP and K2p receptors (acid sensing ion channels, transient receptor potential channels, 2 pore potassium channels) as well as neuropeptides (Pertruska et al., 2002; Rau et al., 2005; Jiang et al., 2006; Rau et al., 2012).

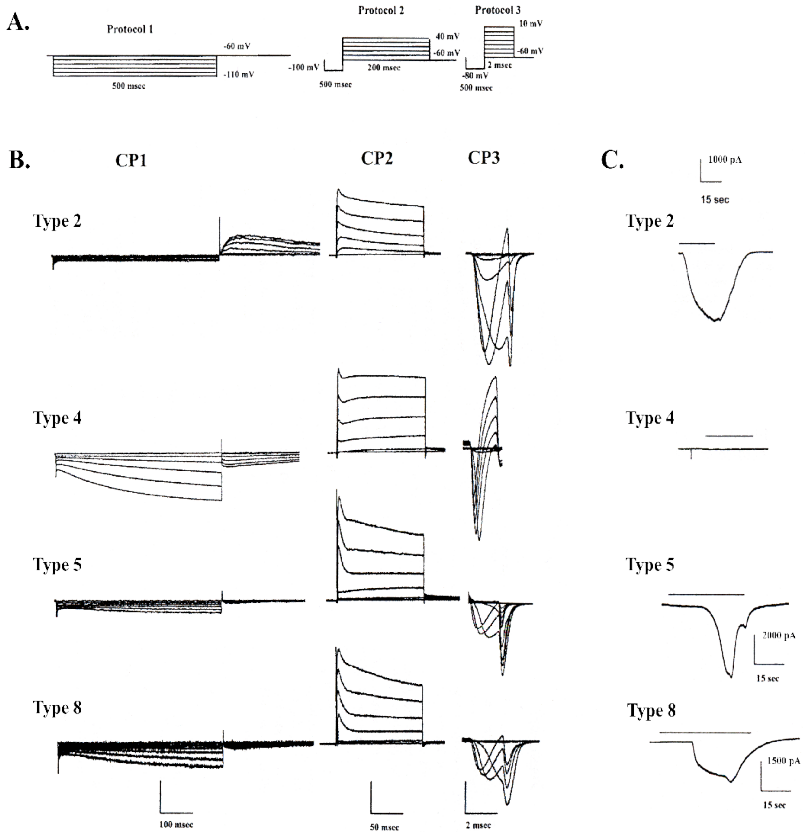

Figure A1. Classification of type 2, 4, 5 and 8 cells by patterns of voltage-activated currents. **A)** Classification protocols 1, 2 and 3 (CP1-3) for cell classification (from Petruska et al., 2000, 2002). **B)** Examples of current signatures for skin (type 2), muscle (type 5) and vascular nociceptors (type 8). Signatures for type 4 (skin nociceptors) are presented for comparison. **C)** Capsaicin (1 μM) response profiles evoked in representative cell types 2, 4, 5 and 8. Vertical scale bars from CP1 represent 500 pA (type 2), 500 pA (type 4), 200 pA (type 5), and 300 pA (type 8). Vertical scale bars from CP2 represent 15,000 pA (type 2), 15,000 pA (type 4), 10,000 pA (type 5), and 10,000 pA (type 8). Vertical scale bars from CP3 represent 5,000 pA (type 2), 10,000 pA (type 4), 15,000 pA (type 5), and 15,000 pA (type 8).

Acute Studies

In the acute studies, we examined how permethrin affected a number of proteins that were expressed in tissue specific nociceptors. This involved isolation a variety of currents in distinct experiments.

Action Potential, Excitability and Spontaneous Activity Experiments. Following classification as a skin, muscle or vascular nociceptor, cells were brought into current clamp mode. The resting membrane potential was identified (<-50 mV excluded from study). A Tyrode solution

containing .001 % ETOH was applied for 2 minutes. Current was injected to bring the cell to -60 mV. Subsequently an action potential was evoked by a brief, high amplitude, current injection (1 ms, 3 nA; 3 replications; 21° C). Excitability was then assessed by a series of stepped current injections. Increasing currents (0.1 to 1 nA) were injected for 250 ms in 10 consecutive steps. Subsequently, a Tyrode solution containing 10 μM permethrin (racemic mixture of 26.4% cis and 71.7% trans in ETOH vehicle; Sigma Aldrich) was applied and excitability and individual action potential were assessed for 10 minutes (1 minute test intervals). The resting membrane potential was adjusted to -60 mV for these tests. If spontaneous activity was detected during the test intervals, excitability testing was terminated and spontaneous activity assessments were begun.

After excitability tests were concluded, the resting membrane adjustments were removed and the cell was permitted to find its natural resting potential. The cell was observed for at least 5 minutes in these conditions in which ETOH or permethrin solutions were continuously applied. In distinct groups of cells, these studies were replicated using: 1) only ETOH vehicle; 2) permethrin + tetrodotoxin (TTX, 1 μM); 3) ETOH vehicle at 35.5° C. When permethrin was applied, we used an 'all glass' superfusion system to avoid binding to plastic tubing (Shafer and Hughes, 2010). The application system is described in the attached manuscript (Jiang et al., 2012; Appendix, p. 65).

Action Potential Measures

Action potential duration. The duration of the action potential was scored as the value (in ms) from the first upswing of the potential above the resting membrane potential until the potential fell below the original resting membrane potential.

Action potential excitability. The number of action potentials evoked during all ten stepped depolarization were counted and totaled.

Spontaneous activity. The number of action potentials observed during the 5 minute observation period were counted. Some of these studies were conducted at 22° C. Others were conducted at 35° C.

Isolation and Characterization of K_v7 Protein Currents.

Voltage activated K^+ channels were characterized in a 'K-Iso' solution containing (in mM) 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-amino pyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with KOH. The reversal potential for K^+ was calculated to be -86 mV. The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

A current subtraction method was used to isolate Kv_7 mediated currents from other K^+ currents that were present as tail currents. The K_v7 voltage deactivation protocol tests were first carried out in the K-Iso solution containing .001% ETOH (pre-applied for 2 min). This was followed by application of the K-Iso solution containing the K_v7 specific antagonist linopirdine (10 μM in ETOH; 2 min application by close superfusion) followed by the K_v7 voltage deactivation protocol. The linopirdine sensitive K_v7 current was isolated by subtraction. The permethrin sensitive K_v7 current was determined by comparisons between cells receiving the sequence: ETOH-ETOH-Linopirdine and ETOH-Permethrin-Linopirdine. The deactivation protocol was repeated following each 2 minute exposure. For the K_v7 deactivation protocol: a 1000 ms step command to -20 mV was followed by a series repolarizing 10 mV steps from -10 to -90 mV (1000 ms; V_H = -60 mV). A tail current could be measured during the repolarization step.

The amplitude of the tail current was measured for 1000 ms from a point 10 ms after the repolarizing voltage step to the point 10 ms prior to the return step to -20 mV. The amplitude of linopirdine sensitive K_v7 current was plotted against the repolarization voltage to obtain a current-voltage relationship.

For contrasts between peak ETOH and permethrin treated currents, we normalized the peak K_v7 linopirdine sensitive currents for cell capacitance (pA/pf), where the cell capacitance is known to be directly proportional to cell volume. The normalized peak current (from -30 mV to -70 mV) was pooled across voltage and tested for significance using a one way ANOVA. A significant 'F' value was followed by contrasts between normalized amplitude at various deactivation

voltages for ETOH and linopirdine treated groups. T-tests were conducted as follow-up tests. The alpha level was set at .05.

Na^+ Current Isolation and Characterization in Acute Studies.

In studies of deactivation and inactivation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-1' solution containing (in mM) 50 NaCl, 92.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH. As needed, 500 nM TTX was added to isolate $Na_v1.8$ from other Nav that were present (Jiang et al., 2011). The pipette solution contained 140 CsF, 10 NaCl, 10 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH. In studies of activation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-2' solution containing (in mM) 30 NaCl, 122.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH.

Acute Studies of $Na_v1.8$ Deactivation. Cells were held at -70 mV. An 8 ms step command to 0 mV was used to evoke a maximal Na^+ current. The cell was repolarized progressively in 10 mV steps ranging from -40 to -70 mV. As 8 ms was insufficient for full inactivation, a tail current could be measured during the repolarization step. In ETOH treated cases, tests were carried out in the Nav-Iso solution containing .001% ETOH. In treated cases, the tests were carried out in the Nav-Iso solution containing 10 μM permethrin in .001% ETOH. Tail currents were fit to exponential decay functions. A time constant, tau_{deact}, was obtained by a successful fit to individual skin, muscle and vascular nociceptors decay currents. The mean and standard deviation of the tau_{deact} were determined by pooling from various cell and treatment conditions. Comparisons were made between the tau_{deact} determined at each voltage step. T-tests for independent groups were used for these comparisons.

The tau_{deact} was subsequently plotted against the deactivation voltage for each cell. An exponential growth function of the form:

$$y = Ae^{(x/k)} + y0$$

was fit to the curve formed from the voltage and decay constant data. A constant *'k'* was determined from a successful fit of this function. These *'k'* values were then used to characterize the voltage dependence of deactivation. The *k* values were pooled by groups and tests were

conducted comparing the ETOH and permethrin treated cells in each nociceptor group. A T-test for independent groups to compare the *k* values. A significant effect was considered to have occurred when $p<0.05$.

Acute Studies of $Na_v1.8$ Activation

The $Na_v1.8$ current was isolated from other Nav by application of the Na-Iso external solution with 500 nM TTX (2 min). In the fluoride based internal solution, the persistent currents ($Na_v1.9$) were inactivated at a holding potential of –70 mV. The remaining, slow, TTXr current could be characterized directly. Using a V_h of –70 mV, in the presence of TTX (500 nM), cells were stepped to 20 mV in 5 mV steps (150 ms duration). In distinct cells, evoked currents were measured in either the presence of .001% ETOH or permethrin (10 μM). The P/4 procedure was applied during data collection to adjust for leak current contributions. All characterizations were performed at room temperature with a series resistance correction of 70-80%.

Individual currents were measured at their peak and transformed into a conductance:

$$G=I_{peak}/(V_m-V_{rev}),$$

where I_{peak} was the test current, V_m the test command voltage, and V_{rev} was calculated from the Nernst equation to be 27.8 mV for Na-Iso-2. The conductance was then normalized to the peak conductance (G_{max}) observed. The voltage dependence of activation was determined from a fit of the voltage-conductance measures to a Boltzman function of the form:

$$G=G_{max}/(1+\exp((V_{.50}-V_m)/K))$$

where $V_{.50}$ is the voltage at which G is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{.5}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). The $V_{.50}$s were then contrasted using two tailed T-Tests. The alpha level was set at .05.

Acute Steady-State Inactivation of $Na_v1.8$. The Na-Iso-1 solution containing 500 nM TTX was used to study the voltage dependence of inactivation of $Na_v1.8$. Tissue specific nociceptive neurons were held at -70 mV. Starting at an inactivation conditioning voltage of -10 mV, progressive command steps were made to -70 mV in 5 mV steps (150 ms). Each of these

commands steps were followed by a fully depolarizing step to 0 mV (150 ms). The peak current evoked at 0 mV was measured and normalized against the maximum evoked current for that cell (I/I_{MAX}). The normalized current was plotted against the conditioning step voltage and fit to a Boltzman type curve of the form:

$I=I_{max}/(1+\exp((V_{.50}-V_m)/K))$

where $V_{.50}$ is the voltage at which I is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{.50}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). The $V_{.50}$ represents the voltage at which half of the $Na_v1.8$ channels are inactivated and K reflects the rate of growth of the relationship. A T-test for independent groups was used to compare the $V_{.50}$ values. A significant effect was considered to have occurred when $p< 0.05$.

Chronic Treatment Studies

In the chronic studies, we examined how permethrin, chlorpyrifos and PB affected a number of proteins that were expressed in tissue specific nociceptors. This involved isolation of a variety of currents in distinct experiments that were similar but not identical to those used in acute studies.

Dosing. Juvenile rats (90-110 g) were used in all studies. Rats were acclimated to the behavioral procedures for 2-5 weeks before dosing began. Animals were treated with neurotoxicants/PB in three regimens (NR1, NR2 and NR3; see Table A1). In NR1 and NR2, neurotoxicants and PB were administered over a 30 days period. During this 30 day period, permethrin (mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), in ETOH, was applied every day, to a shaved area of the back. Chlorpyrifos (Sigma) was applied by subcutaneous injection (corn oil) once every 14 days. PB was given by oral gavage (water) for 14 consecutive days, beginning at day 1. Animals were sacrificed for electrophysiological experiments 4 weeks after the dosing period had ended. Groups of rats consisting of animals receiving equal volumes of ETOH, corn oil and water over the identical time period served as controls. In NR3, the dosages were the same as NR2 but the treatments continued for 60 days and the delays for molecular tests extended to 8-12 weeks.

Table AI

	PB mg/kg, Oral	Permethrin mg/kg, Topical	Chlorpyrifos mg/kg, SC	Exposure days
NR1	13	1.3	60	30
NR2	13	2.6	120	30
NR3	13	2.6	120	60

Permethrin is applied every day

Chlorpyrifos is injected once every 2 weeks

PB is given by gavage every day for 2 weeks every 4 weeks

Action Potential, Excitability and Spontaneous Activity Experiments. Following classification as a skin, muscle or vascular nociceptor, cells were brought into current clamp mode. The resting membrane potential was identified (<-50 mV excluded from study). Current was injected to bring the cell to -60 mV. Subsequently, an action potential was evoked by a brief, high amplitude, current injection (1 ms, 3 nA; 3 replications; 21° C). Excitability was then assessed by a series of stepped current injections. Increasing currents (0.1 to 1 nA) were injected for 250 ms in 10 consecutive steps. After excitability tests were concluded, the resting membrane adjustments were removed and the cell was permitted to find its natural resting potential. The superfusion temperature was then increased to ~35° C (TC_{bip}; Cell Microsytems). Cells were examined for the presence of spontaneous activity over a 3 minute observation period. Measures of action potential duration (APD), action potential afterhyperpolarization duration (AHD), excitability threshold, excitability, resting membrane potential and membrane resistance were made in NR1 and NR2 treated cells.

Action Potential Measures

Action potential duration. The duration of the action potential was scored as the value (in ms) from the first upswing of the potential above the resting membrane potential until the potential again fell below the original resting membrane potential.

Action potential excitability. The number of action potentials evoked during 15 stepped depolarizations were counted and totaled.

Action potential afterhyperpolarization duration. The time in milliseconds from the point in which the repolarizing action potential fell below the previous resting membrane potential until it recovered to within 80% of the previous resting membrane potential.

Spontaneous activity. The number of action potentials observed during a 3 minute observation period were counted.

Isolation and Characterization of K_v7 Protein Currents in Chronic Treatment Studies.

Voltage activated K^+ channels were characterized in a 'K-Iso' solution containing (in mM) 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-amino pyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with KOH. The reversal potential for K^+ was calculated to be -86 mV. The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

A current subtraction method was used to isolate K_v7 mediated currents from other K^+ currents that were present as tail currents. Following application of a K-Iso solution containing .001% ETOH (2 min application), the K_v7 voltage protocol was presented. Subsequently, a K-Iso solution containing the K_v7 inhibitor, linopirdine (10 μM) was presented for 2 minutes. The K_v7 voltage protocol was repeated. Linopirdine treated currents were subtracted from the ETOH treated currents to obtain the linopirdine sensitive, K_v7, current. The total K_{DR} current was also obtained from the linopirdine treated data as the mean of current evoked from 3 consecutive steps to +20 mV. These data were collected 4 weeks after dosing had concluded from rats that

had been neurotoxicant or vehicle treated for 30 days with NR1 or NR2 (Table 1). The voltage protocol was the same as described in acute studies.

The amplitude of the K_v7 tail current was measured for 1000 ms from a point 10 ms after the repolarizing voltage step to the point 10 ms prior to the return step to the deactivation step voltage. Subsequently, the amplitude of linopirdine sensitive K_v7 current was plotted against the repolarization voltage to obtain a current-voltage relationship.

For contrasts between peak ETOH and permethrin treated currents, we normalized the peak K_v7 linopirdine sensitive currents for cell capacitance (pA/pf) where the cell capacitance is directly proportional to cell area. The normalized tail current (from -30 mV to -70 mV) was pooled across voltage and tested for significance using a one way ANOVA. A significant 'F' valued was followed by contrasts between normalized amplitude at various deactivation voltages for ETOH and linopirdine treated groups. T-tests were used as follow-up tests. The alpha level was set at .05.

<u>Na^+ Current Isolation and Characterization in Chronic Treatment Studies.</u>

For assessment of deactivation and inactivation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-1' solution containing (in mM) 50 NaCl, 92.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH. As needed, 500 nM TTX was added to isolate $Na_v1.8$ from other Nav that were present (Jiang et al., 2011). The pipette solution contained 140 CsF, 10 NaCl, 10 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH.

For assessment of activation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-3' solution containing (in mM) 20 NaCl, 122.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH.

<u>Studies of $Na_v1.8$ Deactivation following Chronic Neurotoxicants/PB</u>.

For the deactivation test protocol, cells were held at -70 mV. An 8 ms step command to 0 mV was used to evoke a maximal Na^+ current. The cell was repolarized progressively in 10 mV steps

ranging from -40 to -70 mV. As 8 ms was insufficient for full inactivation, a tail current could be measured during the repolarization step. Following characterization of candidate neurons as muscle, skin or vascular nociceptors, the Nav-Iso-1 solution containing 500 nM TTX was applied, by close superfusion, for 2 minutes. This was continued during the presentation of the deactviation test protocol. All characterizations were performed at room temperature with a series resistance correction of 70-80%.

A time constant, tau_{deact}, was obtained by a successful fit to individual skin, muscle and vascular nociceptors decay currents. The mean and standard deviation of the tau_{deact} were determined by pooling from various cell, vehicle and treatment conditions. Comparisons were made between the tau_{deact} determined at each voltage step. T-tests for independent groups were used for these comparisons.

The tau_{deact} was subsequently plotted against the deactivation voltage for each cell. An exponential growth function of the form:

$$y = Ae^{(x/k)} + y0$$

was fit to the curve formed from the voltage and decay constant data. A constant *'k'* was determined from a successful fit of this function. These *'k'* values were then used to characterize the voltage dependence of deactivation. The *k* values were pooled by groups and tests were conducted comparing the neurons from the chronically neurotoxicant and vehicle treated animals in each nociceptor group. A T-test for independent groups to compare the *k* values. A significant effect was considered to have occurred when the probability was less than 0.05.

Studies of $Na_v1.8$ Activation following Chronic Treatment with Neurotoxicants

The $Nav_{1.8}$ current was isolated from other Nav by application of the Na-Iso-3 external solution in the presence of 500 nM TTX (2 min). Using a V_h of –70 mV, tissue specific nociceptive neurons were stepped to 20 mV in 5 mV steps (150 ms duration; 'activation protocol'). The P/4 procedure was applied during data collection to adjust for leak current contributions. All characterizations were performed at room temperature with a series resistance correction of 70-80%.

Individual currents were measured at their peak and transformed into a conductance:

$G=I_{peak}/(V_m-V_{rev})$, where I_{peak} was the test current, V_m the test command voltage, and V_{rev} was calculated from the Nernst equation to be 16.7 mV for Na-Iso-3. The conductance was then normalized to the peak conductance (G_{max}) observed. The voltage dependence of activation was determined from a fit of the voltage-conductance measures to a Boltzman function of the form:

$$G=G_{max}/(1+\exp((V_{.50}-V_m)/K))$$

where $V_{.50}$ is the voltage at which G is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{.50}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). The $V_{.50}$s were then contrasted using two tailed T-Tests. The alpha level was set at .05.

Studies of Steady-State Inactivation of $Na_v1.8$. Following Chronic Neurotoxicant Treatments. The Na-Iso-1 solution containing 500 nM TTX was used to study the voltage dependence of inactivation of $Nav_{1.8}$. Tissue specific nociceptive neurons were held at -70 mV. Starting at an inactivation conditioning step voltage of -10 mV, progressive command steps were made to -70 mV in 5 mV steps (150 ms). Each of these commands steps were followed by a fully depolarizing step to 0 mV (150 ms; 'inactivation protocol'). The peak current evoked at 0 mV was measured and normalized against the maximum evoked current for that cell during the protocol (I/I_{MAX}). The normalized current was plotted against the conditioning step voltage and fit to a Boltzman type curve of the form:

$$I=I_{max}/(1+\exp((V_{.50}-V_m)/K))$$

where $V_{.50}$ is the voltage at which I is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{.50}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). The $V_{.50}$ represents the voltage at which half of the $Na_v1.8$ channels are inactivated and K reflects the rate of growth of the relationship. A T-test for independent groups was used to compare the $V_{.5}$ values. A significant effect was considered to have occurred when the probability of a type 1 error was less than 0.05.

Studies of $Na_v1.9$ Activation and Deactivation following Chronic Neurotoxicants/PB.

Na^+ Current Isolation and Characterization. Voltage activated Na^+ channels were characterized in a 'TEA bath solution' containing (in mM) 50 NaCl, 112.5 TEA-Cl, 0.1 $CaCl_2$, 0.1 $CdCl_2$ and 10 HEPES, adjusted to pH 7.4 with TEA-OH. The pipette solution contained 140 CsF, 10 NaCl, 5 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH. This is referred to as the 'fluoride internal solution'. Borosilicate pipettes were used (uncoated) and the tip resistance was 2-4 MΩ.

In the fluoride based internal solution, the persistent currents were inactivated at a holding potential of –70 mV. The remaining slow, TTXr current could be isolated and characterized directly. Using a V_h of –120 mV, in the presence of TTX (500 nM), cells were stepped from -80 to -20 mV in 5 mV steps (150 ms duration). The ultraslow inactivating, persistent currents could be fully isolated and characterized by voltage dependence but were well isolated within a voltage range from a V_h of –80 to -45 mV. Positive to -45 mV the presence of $Na_v1.8$ was evident. Therefore all measures of voltage dependent activation to place 150 msec after the activating step. In this manner, any $Na_v1.8$ that may be present would be fully inactivated and only $Na_v1.9$ would be present (Jiang and Cooper, 2011). Voltage dependent deactivation used identical solutions. In this protocol, using a V_h of –70 mV, in the presence of TTX (500 nM), cells were stepped from -70 to -45 (test pulse 150 msec) and subsequently deactivated in 5 mV steps from -50 to -90 mV. All Na_v characterizations were performed at room temperature with a series resistance correction of 60-70%.

For voltage dependent activation individual currents were transformed into a conductance:

$G=I_{peak}/(V_m-V_{rev})$, where I_{peak} was the test current, V_m the test command voltage, and V_{rev} was calculated from the Nernst equation to be 49.6 mV. The conductance was then normalized to the peak conductance (G_{max}) observed. The voltage dependence of activation was determined from a fit of the voltage-conductance measures to a Boltzman function of the form: $G=G_{max}/(1+\exp((V_{.5}-V_m)/K))$, where $V_{.5}$ is the voltage at which G is half maximal, and K is a slope factor.

For the purpose of statistical comparison, voltage-conductance fits were made to each individual cell recording. If cell currents could not be successfully fit, the case was discarded. The $V_{.5}$

determined from a successful fit was used to compare half activation values between different cell populations (Students t-test; α set at .05). This $V_{.5}$ appears in the text and figures. Curves reflecting the voltage dependence of activation were formed by conductance measures that were pooled within each cell class and then fit to Boltzman functions as a group.

The kinetics of activation and deactivation were assessed using current traces evoked at –50 mV (activation) or -45 mV (deactivation). A time constant for activation (tau_{act}) or deactivation (tau_{deact}) was determined by fit of a function of the form: $A_1\exp(-(t-k)/tau_1) + C$, to the rising or falling phase of the current trace over a range representing points from 90% of the peak to 10% of the base current.

Manuscripts and Abstracts

An Abstract Presented to the International Association for the Study of Pain, 2012

Title: The Insecticide Permethrin Activates Muscle but not Skin Nociceptors

Jiang, N., Nutter, T. and Cooper, B.

Aim of Investigation:

Veterans of the 1991 Gulf War report a variety of serious sensory, cognitive and motor deficits. Widespread joint and muscle pain occurred at far higher incidence (~3:1) in Gulf War veterans than in veterans returning from other theaters. The Research Advisory Committee on Gulf War Veterans Illnesses, (GWI; 2008) concluded that exposure to insecticides was a major contributor to GWI. We examined, for the first time, the interaction of the insecticide permethrin with skin and muscle nociceptors.

Methods:

Young male adult rats (90-150 g) were anesthetized and decapitated. Neurons of the dorsal root ganglia were dispersed and plated on 35 mm Petri dishes. Muscle/vascular and skin nociceptors were categorized using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2000, 2002; Rau et. al., 2007). Excitability was assessed, and whole cell patch clamp records were obtained on selected isolated currents before and after exposure to permethrin or alcohol vehicle (10 uM; $Nav_{1.8}$; Kv_7; Ia). All methods were consistent with the Ethical Guidelines for Pain Research.

Results:

Following exposure to permethrin, action potential (AP) duration was greatly increased in type 2 (skin) and type 5 (muscle), and type 8 (skin muscle and putative vascular) nociceptors (6.96 +/- 0.8 vs. 163.7 +/- 44.2, 8.8 +/- 0.7 vs. 105.9 +/- 12.2, and 6.9 +/- 0.6 vs. 82.7 +/- 12.4 ms for types 2, 5 and 8; $p<.001$, n=4-6). No afterhyperpolarization was present. Type 5 muscle nociceptors developed spontaneous activity following exposure to permethrin (34.4 +/- 12.2 APs/5 min permethrin vs. 0.0 APs/5min ETOH; n=5 and 5; $p<.0001$). Application of TTX did not prevent the development of spontaneous activity (TTX 1 uM: 51.6 +/- 27.5 APs/5min; n=6). We subsequently examined the influence of permethrin on currents that could contribute to action potential duration or spontaneous activity. Permethrin (10 uM) greatly increased the tau of deactivation (tau_{deact}) of $Nav_{1.8}$ in all three nociceptor types in a voltage dependent manner (tau_{deact} = 11.3 +/-1.4 vs 5.9 +/- 0.47 ms; type 5, $p<.003$; 11.1 +/- 0.5 vs. 5.9 +/- 0.5 ms, type 8, $p<.001$; 12.1 +/- 1.5 vs. 7.5 +/- 0.3 ms, type 2, $p<.005$; n=6 to 9; at -60 mV). We did not observe any permethrin dependent influences on amplitude or current voltage relationships of Kv_7 (n=8

and 5, type 5); however the amplitude of A-current was significantly increased in permethrin treated type 5 nociceptors (184.6 +/- 30.3 pA vs 437.3 +/- 43.0 pA; n=5 at -30 mV; $p<.02$).

Conclusion:

The insecticide permethrin shifts the tau_{deact} = of $Nav_{1.8}$ in both cutaneous and muscle nociceptors in a manner consistent with influences on action potential duration and the potentiation of multiple AP discharges. However, only one class of muscle nociceptors exhibited spontaneous discharge after exposure to permethrin. Additional, cell specific, currents contribute to the development of spontaneous activity in one class of muscle nociceptor.

Acknowledgements:

Funded by DoD W81XWH-11-1-0453/CDMRP GW100022 to BC. There was no conflict of interest present.

An Abstract Presented at the Society for Neuroscience, November, 2012

Title:

Persistent K^+ channel dysfunction after chronic exposure to insecticides and pyridostigmine bromide

Cooper, B.Y., Nutter, T. J. and Jiang, N.

Introduction: A disproportionate number of soldiers that served in the 1991 Gulf War subsequently developed widespread chronic pain that was not associated with trauma (Stimpson et al., 2006; Thomas et al., 2006). Exposure to insecticides and the nerve gas prophylactic pyridostigmine bromide (PB) were identified as risk factors by the Research Advisory Committee on Gulf War Veterans' Illnesses (GWI). We examined whether synergisms between neurotoxicants/PB could lead to behavioral and molecular indices of chronic pain in the rat.

Methods: Over a period of 30 days, male rats were exposed to two neurotoxicant regimens (NR1 and NR2) composed of chlorpyrifos (60 and 120 mg/kg; SC), permethrin (1.3 and 2.6 mg/kg; topical), and PB (13.0 and 13.0 mg/kg; oral) or their respective vehicles (corn oil, ethanol, water). During and after exposure, we assessed muscle pressure pain thresholds and open field activity. Four weeks after exposures, we used whole cell patch electrophysiology to examine the function of tissue specific DRG nociceptor proteins expressed in skin, muscle, and putative vascular nociceptors.

Results: Rest times increased during NR1 and NR2 but subsided when exposure ceased ($p<.001$ and .03; n=23 and 9). Muscle pain thresholds were decreased during, but not after, NR2 treatment only ($p<.04$, n=6 and 12). Four weeks after treatments, K_{DR} and K_v7 currents were significantly altered by NR1 and NR2 ($p <.04$-.04 and.002-.008; n= 6-10). The voltage dependence of $Na_v1.8$ (inactivation, deactivation), spontaneous activity or action potential excitability were unchanged. The significant increases (K_{DR}, NR1 and NR2; K_v7 in NR2) and decreases (K_v7 in NR1) in the normalized amplitude of channel currents were only present in putative vascular nociceptors; muscle and skin nociceptors were unaffected.

Conclusions: Putative vascular nociceptors manifested special vulnerability to chronic insecticide exposure. Although various behavioral and molecular changes were not all consistent with a pain condition, the persistent molecular alterations and unique susceptibility of this nociceptor population could be important for the etiology of GWI pain.

Support: Funded by DoD W81XWH-11-1-0453/CDMRP GW100022 to BC

Abstracts Submitted:

An Abstract Submitted to the Society for Neuroscience, November, 2013
Cooper, B.Y. and Nutter, T.J.

Persistent modification of $Na_v1.9$ following chronic exposure to insecticides and pyridostigmine bromide

Introduction. Many veterans of the 1991 Gulf War (GW) returned from the conflict with widespread chronic pain. Exposure to insecticides and other agents may have contributed to these symptoms. Pyrethroid insecticides target the primary action potential forming voltage activated Na^+ channel protein expressed in mammalian nociceptors ($Na_v1.8$). In prior studies, we have shown that there is little indication that chronic exposure to, permethrin, a pyrethroid that was used extensively by deployed soldiers, in combination with other GW relevant agents (chlorpyrifos, pyridostigmine bromide) have any lasting influence on $Na_v1.8$ (voltage dependent activation, inactivation, deactivation and amplitude). In these studies, we examined whether any delayed effects of these agents could be observed in nociceptor $Na_v1.9$.

Methods. Young adult male rats were exposed to permethrin (2.6 mg/kg; topical), chlorpyrifos (120, mg/kg; s.c.) and pyridostigmine bromide (13 mg/kg; gavage) or associated vehicles for 60 days (n=26). Eight weeks after treatment ended, rats were euthanized, dorsal root ganglia were excised, and neurons plated on 35 mm petri dishes. Whole cell patch experiments were conducted on muscle and putative vascular nociceptors. Muscle pain thresholds and open field activity were also assessed.

Results. During exposure to neurotoxicants, rats exhibited lowered muscle pain thresholds (61.2 +/- 1.7 vs 68.9 +/- 2.6 g; $p<.05$) and increased activity; but these measures returned rapidly to normal levels when treatments ended (n=26). Resting periods remained prolonged both during and 8 weeks after exposure ($p<.05$). Eight weeks after treatment ended, voltage dependent activation of $Na_v1.9$ was unchanged in muscle and vascular nociceptors ($V_{.50}$ = -49 to -52 mV; n=27); however, in vascular nociceptors, we did observe a significant increase in peak amplitude (20.0 +/- 2.2 vs 13.9 +/- 1.7 pA/pF; n=8) and significant shifts in the tau_{act} (10.9 +/- 2.5 vs 5.8 +/- 0.5 msec; n=6 and 8; $p<.04$) and tau_{deact} of $Na_v1.9$ (2.0 +/- 0.13 vs 2.65 +/- 0.11 msec; n=5 and 9). Although the tau_{act} of muscle nociceptor $Na_v1.9$ was also significantly increased (14.1 +/- 2 vs 7.6 +/- 1.3; $p<.04$, n= 9 and 6), none of the other influences were observed in skin or muscle nociceptors.

Conclusions. Chronic exposure to insecticides produced long term changes in the amplitude and kinetics of nociceptor $Na_v1.9$. The relationship to chronic pain in Gulf War veterans is unclear, but an increase in $Na_v1.9$ amplitude might lead to increased excitability.

Support: Funded by DoD W81XWH-11-1-0453/CDMRP GW100022 to BC

DoD Related Abstract

There were no DoD related symposia scheduled during the reporting period

Manuscripts

Two manuscripts are attached below

1) The Molecular and Cellular Interactions of Permethrin with Mammalian Nociceptors at Physiological Temperatures

   This manuscript was published in *Neuro Toxicology*

2) Persistent Na^+ and K^+ Channel Dysfunctions after Chronic Exposure to Insecticides and Pyridostigmine Bromide

   This manuscript was submitted to *Neuro Toxicology*

The Molecular and Cellular Interactions of Permethrin with Mammalian Nociceptors at Physiological Temperatures

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Abstract

The influence of pyrethroid insecticides is thought to be abrogated at mammalian physiological temperatures. Yet there are many reports of transient painful and paresthesia following accidental exposures. Using whole cell patch clamp techniques, we examined the interaction of the pyrethroid insecticide permethrin on skin, muscle and putative vascular nociceptors of the rat DRG (dorsal root ganglion) was increased in all nociceptor populations, but only muscle nociceptors developed spontaneous activity or increased excitability (tests at 21C). TTX (tetrodotoxin) did not prevent the development of spontaneous activity or reduce excitability. We examined the influence of permethrin on TTX resistant channel proteins that control excitability and spontaneous activity ($Na_v1.8$, voltage-gated sodium channel 1.8; K_v7, voltage gated potassium channel 7). In all nociceptor populations, permethrin increased the tau of deactivation (tau_{deact}), in a voltage dependent manner, and hyperpolarized the $V_{1/2}$ for activation over 10 mV. There were no permethrin dependent influences on K_v7, or on the voltage dependence of inactivation of $Na_v1.8$. The influence of permethrin on AP duration, after hyperpolarization, spontaneous activity, half-activation potential ($V_{1/2}$) and tau_{deact} were reduced, but not fully reversed, when tests were conducted at 35 C. In conclusion, permethrin greatly modifies the voltage dependent activation and deactivation of $Na_v1.8$ expressed in skin, muscle and vascular nociceptors. These influences remain significant at 35 C. One population of muscle nociceptors exhibited a unique vulnerability to the acute administration of permethrin manifested as increased excitability and spontaneous activity.

1.1 Introduction

In humans, burning and stinging sensations have been reported following either ingestion (Gotoh, et al., 1998) or acute topical contact with the type I pyrethroid insecticide, permethrin (Kolmodin-Hedman et al., 1982; Tucker and Flannigan, 1983; Flannigan and Tucker, 1985) or other pyrethroid formulations (Knox et al., 1984; Wilks, 2000; see also Wolansky and Harrill, 2008). Specific pyrethroids (permethrin and allethrin) have also been linked to the development of certain chronic pain states (Binns et al., 2008). The influence of type I and type II pyrethroids (allethrin, tetramethrin, deltamethrin) on TTXs and TTXr Na_v proteins of the DRG suggest a relatively direct pathway to nociceptor activation and pain (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996; Song and Narahashi, 1996a,b; Tabarean and Narahashi, 1998; Tabarean and Narahashi, 2001). However, the apparent influence of pyrethroids on mammalian nociceptors has not been directly demonstrated, and stands in contrast with the supposed mitigating influences of body temperature and other factors that make it safe for use near humans.

It is well established that both type 1 and type 2 pyrethroid insecticides alter the physiology of voltage-gated sodium channels (VGSC, or Na_v). While there have been few studies on the influence of the type 1 pyrethroid, permethrin, on neural channel protein function, the influence of structurally similar pyrethroid neurotoxicants have been studied extensively. In insects, pyrethroids induce or obstruct neural activity via modulation of activation, steady-state inactivation and deactivation of Na_v proteins (Narahashi et al., 1998; Narahashi, 2000; Ray and Fry, 2006; Soderlund, 2010). Early investigations of interactions with sensory neurons of the DRG (dorsal root ganglion), confirmed powerful influences between several pyrethroids (allethrin, tetramethrin, deltamethrin) and structurally similar mammalian Na_v (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996; Tabarean and Narahashi, 1998; Tabarean and Narahashi, 2001). Altered activation, steady-state inactivation and deactivation of Na_v proteins have been reported across the diverse family of mammalian TTXs (tetrodotoxin sensitive) and TTXr (tetrodotoxin resistant) Na_v (e.g., $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, $Na_v1.8$), but the specifics of modulation by type I and type II pyrethroids differed both qualitatively and quantitatively (Song et al., 1996; Smith and Soderlund, 1998; Motomura and

Narahashi, 2001; Dekker et al., 2005; Choi and Soderlund, 2006; Meacham et al., 2008; Breckenridge et al., 2009; Tan and Soderlund, 2009; Tan and Soderlund, 2010).

Mammals are highly resistant to the toxic influences of pyrethroids. The absence of lethal effects on mammals is believed to be due, in part, to a powerful temperature dependence of pyrethroid actions on Na_v that substantially reduces their influence at body temperature. Other factors such as body size, catabolic rates and innate sensitivity also contribute to large safety factors associated with pyrethroids (Soderlund, 2002; Bradberry et al., 2005; Ray and Fry, 2006). While some temperature-dependent effects of pyrethroids have been documented, many aspects of these interactions have not been examined. Using a preparation of central nervous system neurons that expressed the fast inactivating TTXs Na_v channel proteins, Song and Narahashi demonstrated that the slowing of TTXs Na_v deactivation by tetramethrin was not completely reversed at 35° C (Song and Narahashi, 1996b). Other important pyrethroid influences (voltage dependent activation and inactivation) were not examined at elevated temperatures. Moreover, the action potentials of mammalian nociceptors are formed by TTXr $Na_v1.8$ (Djouhri et al., 2003a,b; Jiang and Cooper, 2011). The thermal dependence of pyrethroids, on TTXr channels, has never been examined at physiological temperatures. At 28° C, allethrin retains the capacity to alter TTXr deactivation, but also exhibits other temperature independent modifications of $Na_v1.8$ physiology (Ginsberg and Narahashi, 1999).

Accordingly, we initiated a series of studies to examine how permethrin differentially interacted with specific skin, muscle and putative vascular nociceptors that express the pyrethroid sensitive TTXr protein $Na_v1.8$. Experiments were conducted at both room and physiological temperatures. We observed that permethrin had predictable influences on $Na_v1.8$ across superficial and deep nociceptor families, but that the cellular consequences of this interaction differed. Moreover, substantial influences of permethrin on voltage dependent activation, deactivation and action potential characteristics were still evident at 35° C.

2.1 Methods

2.2 Preparation of Cells. Young adult male Sprague-Dawley rats (n=95; 90-110 g) were anesthetized (Isoflurane) and euthanized by decapitation. The dorsal root ganglia were excised, digested in a solution containing type 1 collagenase and Dispase II. The procedure has been described in detail previously (Petruska et al., 2002). Isolated neurons were plated on 8-10, 35 mm Petri dishes. Neurons were bathed continuously in Tyrode's solution, containing (in mM) 140 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. Electrodes were formed from boroscilate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm. Recordings were completed within 2-10 h after plating. Only one cell was used per Petri dish. Studies were conducted at room temperature unless otherwise noted. All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee.

2.3 Recording and Characterization of Skin, Muscle and Vascular Nociceptors. Whole cell recordings were made with an Axopatch 200B or Multiclamp 700 (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and Digidata 1322A (Molecular Devices). Series resistance (R_s) was compensated 65-70% with Axopatch compensation circuitry. Whole cell resistance and capacitance were determined by the Clampex software utility. Recorded currents were sampled at 10-20 kHz and filtered at 2 kHz (Bessel filter).

Classification of nociceptors into 'types' was performed by presentation of a series of depolarizing or hyperpolarizing characterization protocols originally developed by Scroggs and extended by Cooper (Cardenas et al., 1995; Petruska et al., 2000; Petruska et al 2002). All reported data were derived from recordings made on type 2 (C nociceptor), type 5 (C or type IV nociceptor), and type 8 (Aδ or type IV nociceptor). Type 2 and type 5 were identified as skin and muscle nociceptors, respectively, in tracing studies (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2012). Type 8 nociceptors were traced from all tissue sites (skin, muscle, colon, mucosa; Rau et al., 2007; Rau et al., 2012) and are presumed to be vascular in origin.

2.4 Permethrin Application. Agents were applied by close superfusion (sewer pipe). An all glass superfusion system was devised to avoid complications due to the known binding of highly lipophilic pyrethroids to plastics (Tatebayashi and Narahashi, 1994; Shafer and Hughes, 2010). Ten (10) ml glass syringe reservoirs were coupled to a small Plexiglas manifold by a flexible glass tubing system terminating in a 'sewer pipe' that was positioned within 2 mm of the targeted cells. The glass tubing consisted of 3 rigid sections of 150 mm glass (.86 mm inner diameter, 1.5 mm outer diameter; Sutter Instruments). Each rigid piece was linked by a 3 mm section of silastic tubing. Only about 1 mm of each silastic linker was in contact with the permethrin containing solution. The Plexiglas manifold was not exposed to permethrin until the actual application occurred. Following each application of permethrin, the manifold was rinsed for at least 2 minutes with 100 % ETOH. At the termination of each experiment, the plastic toggle that was exposed to permethrin was discarded and flexible glass tubing was rinsed with 10 ml of 100% ETOH. To equalize flow rates, the glass application system was used to apply all agents. Stock solutions of permethrin were prepared and maintained in glass bottles (racemic mixture of 26.4% cis and 71.7% trans in ETOH vehicle; Sigma Aldrich). In studies in which heated solutions were used, a feedback controlled bath controller (Model TC_{bip}) and Model HPRE applicator (Cell MicroControls, Norfolk, VA) was used to pre-heat the superfused solution to the desired temperature.

2.5 Action Potential Duration, Excitability and Spontaneous Activity Experiments. In current clamp mode, an action potential was evoked by a brief, high amplitude, current injection (1 ms, 3 nA; 3 replications). Action potential duration was measured as the width of the action potential at its base (Petruska et al., 2002). Excitability was assessed by a series of stepped current injections (0.1 to 1 nA; 250 ms; 10 consecutive steps). Excitability was quantified as the total number of action potentials evoked in the 10 stepped current injections. The resting membrane potential was adjusted to -60 mV for the excitability and action potential duration tests. Subsequently, a Tyrode solution containing 10 μM permethrin or .001% ETOH was applied and excitability and individual action potential characteristics were assessed for 10 minutes (1 minute test intervals). If spontaneous activity was detected during the test intervals, excitability testing was terminated and spontaneous activity assessments were begun. After excitability tests were

concluded, the resting membrane adjustments were removed and the cell was permitted to find its natural resting potential. The cell was observed for at least 5 minutes under these conditions in which ETOH or permethrin solutions were continuously applied.

2.6 Na^+ Current Isolation and Characterization. In studies of deactivation and inactivation, voltage activated Na^+ channels were characterized in a solution designated as 'Nav-Iso-1' containing (in mM) 70 NaCl, 92.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH. As needed, 500 nM TTX was added to isolate $Na_v1.8$ from other Na_v that were present (Jiang and Cooper, 2011). The pipette solution contained 140 CsF, 10 NaCl, 10 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH. In studies of activation, voltage activated Na^+ channels were characterized in a solution designated as 'Nav-Iso-2' containing (in mM) 40 NaCl, 112.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH.

2.7 $Na_v1.8$ Deactivation. Cells were held at -70 mV. An 8 ms step command to 0 mV was used to evoke a maximal Na^+ current. The cell was repolarized progressively in 10 mV steps ranging from -40 to -70 mV. As 8 ms was insufficient for full inactivation, a tail current could be measured during the repolarization step. In vehicle cases, tests were carried out in the Nav-Iso-1 solution containing .001% ETOH. In treated cases, the tests were carried out in the Nav-Iso-1 solution containing 10 μM permethrin in .001% ETOH. Both solutions contained 500 nM TTX. The P/4 procedure was applied during data collection to adjust for leak current contributions. Following experiments, $Na_v1.8$ tail currents were fit to exponential decay functions. A time constant, tau_{deact}, was obtained by a successful fit to decay currents of individual skin, muscle and vascular nociceptors. The mean and standard deviation of the tau_{deact} were determined by pooling from various cell and treatment conditions. ANOVA was performed on the Treatment x Voltage data for each tissue nociceptor class. A significant interaction was followed with individual comparisons between the permethrin and ETOH tau_{deact} at each voltage step. The Bonferroni correction procedure was used to protect against type 1 error ($p<.009$).

2.8 $Na_v1.8$ Activation. The $Na_v1.8$ current was isolated from other Na_v by application of the Na-Iso-2 external solution with 500 nM TTX (2 min). In the fluoride based internal solution, the persistent currents ($Na_v1.9$) were inactivated at a holding potential of –70 mV (Jiang and Cooper, 2011). The remaining, slow, TTXr (TTX resistant) current could be characterized directly.

Using a V_h of –70 mV, in the presence of TTX (500 nM), cells were stepped to 20 mV in 5 mV steps (150 ms duration). In distinct cells, evoked currents were measured in either the presence of .001% ETOH or permethrin (10 μM in ETOH). The P/4 procedure was applied during data collection to adjust for leak current contributions. All characterizations were performed at room temperature unless otherwise noted. Liquid junction potentials errors were not corrected.

Individual currents were measured at their peak and transformed into a conductance:

$G=I_{peak}/(V_m-V_{rev})$,

where I_{peak} was the peak current evoked by a given step command, V_m the corresponding voltage step applied, and V_{rev} was calculated from the Nernst equation to be 27.8 mV for Na-Iso-2. The conductance was then normalized to the peak conductance (G_{max}) observed. The voltage dependence of activation was determined from a fit of the voltage-conductance measures to a Boltzman function of the form:

$G=G_{max}/(1+\exp((V_{1/2}-V_m)/K))$

where $V_{1/2}$ is the voltage at which G is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{1/2}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). The $V_{1/2}$ values were then compared using two tailed Student's T-Tests. The alpha level was set at .05.

2.9 Steady-State Inactivation of $Na_v1.8$. The Na-Iso-1 solution containing 500 nM TTX was used to study the voltage dependence of inactivation of $Na_v1.8$. Starting at an inactivation conditioning voltage of +10 mV, progressive command steps were made to -70 mV in 5 mV steps (400 ms). Each of these commands steps were followed by a fully depolarizing step to 0 mV (150 ms). The peak current evoked at 0 mV was measured and normalized against the maximum evoked current for that cell (I/I_{MAX}). The normalized current was plotted against the conditioning step voltage and fit to a Boltzman type curve:

$I=I_{max}/(1+\exp((V_{1/2}-V_m)/K))$

From the curve fits made to each individual cell, a $V_{1/2}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). Student's T-test for independent groups was

used to compare the $V_{1/2}$ values. A significant effect was considered to have occurred when $p < 0.05$.

2.10 Isolation and Characterization of K_v7 Channel Currents. K_v7 mediated tail currents were characterized in a solution designated as 'K-Iso' containing (in mM) 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-amino pyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with KOH. The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

A current subtraction method was used to isolate K_v7 mediated currents from other K^+ currents that were present as deactivation tail currents. For the K_v7 deactivation protocol: a 1000 ms step command to -20 mV was followed by a series of repolarizing 10 mV steps from -10 to -90 mV (1000 ms; V_H = -60 mV) followed by a return step to -20 mV. A tail current could be measured during the repolarization step.

The K_v7 voltage deactivation protocol tests were first carried out in the K-Iso solution containing .001% ETOH (pre-applied for 2 min). This was followed by application of the K-Iso solution containing the K_v7 specific antagonist linopirdine (10 μM in ETOH; 2 min application by close superfusion) followed by the K_v7 voltage deactivation protocol. The linopirdine sensitive K_v7 current was isolated by subtraction. The permethrin sensitive K_v7 current was determined by comparisons between cells receiving the sequence: ETOH-ETOH-Linopirdine and ETOH-Permethrin-Linopirdine.

The amplitude of the tail current was measured from a point beginning 10 ms after the repolarizing voltage step (-30 to -90 mV) to the point 10 ms prior to the return step to -20 mV (see figure 8). The amplitude of linopirdine sensitive K_v7 current was plotted against the repolarization voltage to obtain a current-voltage relationship. As described above, a Boltzman function was fit and a $V_{1/2}$ determined for each individual cell. Student's T-test was used to compare the pooled $V_{1/2}$ values for ETOH and permethrin treated cells. The normalized amplitude of the linopirdine sensitive current was determined as the measured amplitude (pA) divided by the cell size parameter (pF). The normalized amplitudes were pooled across

deactivation voltages (-30 to -70 mV) to obtain a mean normalized current. Student's T-tests were performed as described above.

2.11 Immunohistochemistry. In certain cases, the electrode was removed from the cell surface and the approximate position of the recorded cell was marked on the glass below the field location. The bath solution was replaced with 4% paraformaldehyde (PFA) in phosphate – buffered saline (PBS) for 20-30 min and then replaced with a similar solution containing 0.4% triton X-100. Fixed cells were kept refrigerated prior to the labeling. For labeling studies we followed procedures as described in (Petruska et al., 2002). Concentration of primary antisera for potassium channel KCNQ3 was optimal at 1:2000; Millipore, Billerica, MA). The secondary antisera was used at a concentration of (1:1000 Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L); Life Technologies Corporation, Carlsbad, CA). The cells were viewed with an inverted Nikon TE2000-U (Melville, NY) microscope equipped with appropriate fluorescence filters. The controls for possible independent binding of all secondary antisera were negative (n=8).

3.1 Results

3.2 Action Potential Features and Excitability. Studies were conducted on skin (type 2), muscle (type 5) and type 8, vascular nociceptors (Rau et al., 2007; Rau et al., 2012). Cells were brought into current clamp mode and the influence of permethrin (10 μM) on nociceptor action potentials was assessed. Consistent with the influence of the type II pyrethroid, deltamethrin (Tabarean and Narahashi, 1998), the type I pyrethroid, permethrin, significantly increased action potential duration. Skin, muscle and vascular nociceptors were affected in a similar manner (figure 1). The increase in duration was remarkable and so extensive as to completely obscure the AHP period (6.96 +/- 0.8 vs 163.7 +/- 44.2; 8.8 +/- 0.7 vs. 105.9 +/- 12.2, and 6.9 +/- 0.6 vs. 82.7 +/- 12.4 ms for types 2, 5 and 8; $p<.001$, n=4-6; figure 1).

We assessed whether permethrin-induced changes in AP characteristics would be associated with altered membrane excitability. Current was injected to maintain an RMP (resting membrane potential) of -60 mV. A series of current injections were then performed on ETOH and

permethrin treated nociceptors (10 current injection steps of 0.1-1.0 nA; 225 ms/test). Over an observation period of 10 minutes (1 min test intervals), the number of action potentials evoked during permethrin exposure significantly increased in type 5 muscle nociceptors (13.0 +/- 4.5 (perm) vs 1.0 +/- 2.2 (ETOH) APs; n=5 and 6; figure 2a and 2b) but not in skin or vascular nociceptors. Despite correction of the RMP to -60 mV, the AP threshold was also significantly lowered for type 5 muscle nociceptors (-.187 +/- 0.07 vs 0.10 +/- 0.01 nA, $p<.04$, n = 8 and 6, permethrin and ETOH treated, respectively; data not shown).

Place figure 1 about here

With the application of permethrin, all type 5 muscle nociceptors depolarized and exhibited spontaneous discharge (34.4 +/- 12.2 APs/5 min permethrin vs. 0.0 APs/5min ETOH; figure 2c and 2d). This activity sometimes took the form of high frequency bursts that were interrupted by periods of inactivity; more often, AP discharges were single APs separated by relatively long intervals (see figure 2c). Activity typically began at -42.4 +/- 4.2 mV (n=6). In contrast, despite gradual depolarization of all cells under these conditions, neither vascular nor skin nociceptor populations exhibited spontaneous activity following permethrin application (n=16). ETOH vehicle did not produce spontaneous activity in any nociceptor class over equivalent application periods (n=8). We initiated studies to identify the molecular basis of spontaneous activity in muscle nociceptors.

To examine whether TTXs (TTX sensitive) currents were essential to the development of spontaneous activity, we exposed a group of type 5 muscle nociceptors to permethrin in the presence of TTX (10 and 1 μM, respectively; n=6). Using identical procedures as above, 5 of 6 treated nociceptors exhibited spontaneous activity in the presence of permethrin+TTX (51.6 +/- 27.5 APs/5min; n=6; figure 2d). All TTX treated cases also exhibited increased excitability (figure 2b). Action potential duration was not significantly reduced by co-application of TTX (105.9 +/-12.2 vs. 115.0 +/- 6.1 ms with TTX (not shown). Nor was there a difference in the RMP at which activity began (-41.5 +/- 1.5 mV, n=5). We can conclude from these studies that

TTXr Na_v proteins (TTX resistant), such as $Na_v1.8$, were sufficient to maintain permethrin influences on AP duration, excitability and spontaneous activity of muscle nociceptors.

Place figure 2 about here

3.3 Voltage Dependent Activation, Inactivation and Deactivation of $Na_v1.8$. Permethrin exerted unique cellular influences on certain classes of deep tissue nociceptors. We began a series of studies to determine the molecular basis of the excitability and spontaneous activity exhibited by type 5 nociceptors. Muscle nociceptors, as well as many other nociceptor types, express the pyrethroid sensitive TTXr $Na_v1.8$ channel protein (Djouhri et al., 2003a,b; Amir et al., 2006; Jiang and Cooper, 2011). Accordingly, we applied protocols to examine the activation, inactivation and deactivation of $Na_v1.8$ in the presence of permethrin. These outcomes were contrasted with other superficial and deep nociceptor populations that had not manifested spontaneous discharges.

The production of action potentials (excitability) is partly dependent on Na_v channel activation. We examined whether the activation of $Na_v1.8$ was differentially influenced in deep and superficial nociceptors. Skin, muscle and vascular nociceptors were identified in the usual manner. A Na-Iso-2 solution was used to separate Na^+ currents from other voltage sensitive membrane currents. Permethrin was applied for 2 minutes. Subsequently, a $V_{1/2}$ was derived from Boltzman functions fit to the normalized voltage-conductance (G) plots. Statistical comparisons were then made between the $V_{1/2}$ of ETOH/ETOH treated cases and ETOH/Permethrin treated cases. Consistent with increased excitability of muscle nociceptors, permethrin shifted the midpoint of excitation ($V_{1/2}$) more than 10 mV in the hyperpolarizing direction (figure 3b). No significant excitation shifts were observed in ETOH/ETOH treated cases (not shown; n=19). Although excitability changes were limited to muscle nociceptors, large hyperpolarizing, statistically similar, shifts in $V_{1/2}$ were observed in skin and vascular nociceptors as well. No changes in normalized peak amplitude were observed in any nociceptor class (step to 0 mV; data not shown). Accordingly, differential influences of permethrin on

excitability could not be accounted for by differential actions on activation of $Na_v1.8$ in superficial and deep nociceptors.

Place figure 3 about here

The prolongation of action potential duration and the absence of an AHP (figure 1) could have promoted repetitive, spontaneous, discharges in muscle nociceptors. Type I pyrethroids alter deactivation tail currents of Na_v channels (Soderlund, 2010). The slowing of $Na_v1.8$ deactivation could contribute to the differential outcome in superficial and deep nociceptors. We directly assessed the influence of permethrin on the deactivation of nociceptor $Na_v1.8$ in three classes of nociceptors. Deactivation time constants (tau_{deact}) were determined by fits of exponential decay functions to Na^+ currents that were terminated 8 msec after a strong depolarizing voltage step (step to 0 mV). Permethrin treated skin, muscle and vascular nociceptors exhibited significant increases in the deactivation time constants consistent with a prolongation of action potential duration (e.g., tau_{deact} = 11.3 +/-1.4 vs 5.9 +/- 0.47 ms at -60 mV; type 5 muscle, $p<.01$), but we did not observe significant differences between superficial and deep nociceptor groups (figure 4).

Insert figure 4 about here

Additional tests were performed to assess whether a neurotoxicant would alter the steady state, fast, inactivation of nociceptor $Na_v1.8$. A $V_{1/2}$ for inactivation was determined from Boltzman function fits to currents that were released from inactivation by stepped reductions of conditioning voltages. Muscle, skin and putative vascular nociceptors were exposed to either ETOH or permethrin. Two minutes following application, we were unable to demonstrate any influence on the voltage dependence ($V_{1/2}$); however, time constants fit to the decay phase of the evoked current (tau_{inact}) were profoundly increased by permethrin. As with activation and

deactivation, there were no differences between the tau_{inact} determined in superficial and deep nociceptors (figure 5).

place figure 5 about here

Although the lethal influences of pyrethroids are manifested via Na_v channel proteins, it is known that pyrethroids can also modulate function of other neuronal proteins (Ray and Fry, 2006; Clark and Symington, 2011). Activation of K_v7 channel proteins (classically designated as M-current or I_M) opposes the initiation of action potentials. A decline in K_v7 activity could have a substantial influence on neuronal excitability and spontaneous discharge (Marrion, 1997; Robbins, 2001). In order to understand how permethrin initiated spontaneous activity in muscle nociceptors, we examined whether K_v7 proteins were influenced by the acute application of permethrin.

Skin, vascular and muscle nociceptors differed with respect to permethrin-induced transitions to spontaneous activity (figure 2). We isolated type 2, type 5 and type 8 nociceptors to determine if they expressed K_v7 mediated currents. The K-iso solution containing .001% ETOH was applied during presentation of a protocol designed to isolate deactivating K_v7 currents (see figure 6). ETOH or permethrin was applied for 2 min, followed by the K_v7 specific inhibitor linopirdine or linopirdine+permethrin (type 2 and 5 only). Linopirdine sensitive K_v7 currents were identified by subtraction. Type 2 skin nociceptors did not express significant linopirdine sensitive current. Substantial K_v7 current was present in vascular and muscle nociceptors, but we were unable to identify any influence of permethrin on K_v7 amplitude or voltage dependence in tests on muscle nociceptors (figure 6). Immunocytochemistry was subsequently performed on both type 5 (n=6) and type 8 (n=6) nociceptors. All cases were found to be positive for $K_v7.3$ protein (figure 6b). Control tests on identified cells were all negative (n=8; secondary alone; data not shown).

Place figure 6 about here

3.4 Permethrin Induced Shifts In Nav1.8 Persist at Mammalian Physiological Temperatures.

Humans are highly resistant to pyrethroid poisoning. At 35° C, some of the actions of pyrethroids on the mammalian central nervous system TTXs Na_v are greatly diminished or abrogated (Song and Narahashi, 1996b). Nevertheless, it is well documented that exposure to permethrin formulations causes pain in humans lasting minutes to hours (Soderlund et al., 2002; Ray and Fry, 2006). These sensory events are modulated, not by TTXs, but by TTXr $Na_v1.8$. To the extent that the influences of permethrin are not reversed at normal body temperatures, persistent sub-lethal actions of pyrethroids could lead to maladaptations in human nociceptive sensory neurons. Accordingly, we examined the extent to which the capacity of permethrin to modify the physiology of $Na_v1.8$ was reduced at 35° C.

Following isolation of types 2, type 5 or type 8 nociceptors, action potentials were evoked as previously described (3 nA, 1 msec, 3 replications). After preliminary characterization in a Tyrode's solution containing ETOH (.001%), permethrin (10 μM) was applied continuously for 10 minutes. APs were then evoked at 21° and 35° C (2 min at elevated temperature). Nociceptors were then observed for 5 minutes for the development of spontaneous activity at 35° C.

As expected, the permethrin-induced prolongation of action potential duration and abrogation of AHP were attenuated at elevated temperatures, but neither recovered completely from the influence of permethrin. Although AP duration was greatly reduced at 35° C, the permethrin treated neurons retained a significantly longer AP duration than the ETOH treated group (figure 7a). In the same permethrin treated neurons, the AHP at 35° C was still less than half of its pre-permethrin level in skin, muscle and vascular nociceptors (figure 7b, c and d; $p<.004$). Muscle nociceptors had been consistently activated in the presence of permethrin at room temperature, but they exhibited far less spontaneous activity near physiological temperature (figure 7e; van den Bercken et al., 1977; see also Motomura and Narahashi, 2000). At 35° C, one type 8 nociceptor did exhibit spontaneous activity after permethrin treatment (data not shown; n=7), but there was no spontaneous activity in type 2 skin nociceptors in these conditions (n=5).

Place figure 7 about here

We used similar methods to examine the molecular basis of the rescue of action potential duration. Studies were conducted on skin nociceptor $Na_v1.8$. Superfusion temperature was increased to 35° C and the deactivation protocol was applied in the presence of ETOH (.001%) or permethrin (10 μM; 2 min). Following tests at physiological temperature, the superfusion solution temperature was reduced to 21° C (ETOH). As observed above, the effect of permethrin was greatly reduced at 35° C; however, a statistically significant slowing of deactivation clearly remained (figure 8). When superfusion temperatures were shifted to 21° C, the expected pronounced permethrin effects were readily visible. The substantial improvement of $Na_v1.8$ deactivation at 35° C would greatly contribute to the partial restoration of action potential duration. However, as a complete reversal did not occur, it is clear that a persistent failure to fully achieve normal $Na_v1.8$ deactivation could be a consequence of human exposure to permethrin, and, following prolonged exposure might lead to persistent CNS and/or PNS maladaptations (Binns et al., 2008).

At room temperature, permethrin greatly shifted the $V_{1/2}$ for $Na_v1.8$ activation in all nociceptor populations (figure 3). Certainly this may have contributed to the development of spontaneous activity. For tests at elevated temperatures, skin nociceptors were isolated and experiments were conducted as previously described. As we observed with other cellular and molecular measures, temperature greatly reduced the influence of permethrin ($p<.005$; 21° vs 35° C), but significant shifts in the $V_{1/2}$ for activation were retained at 35° C ($p<.0002$; figure 8). As a result, an increased propensity to discharge would be expected in mammalian neurons chronically exposed to permethrin.

Place figure 8 about here

4.1 Discussion

Our studies represent the first systematic attempt to determine how pyrethroids acutely interact with identified nociceptor subpopulations that innervate superficial or deep tissues, and the first attempt to quantify the full range and degree of pyrethroid modulation of Na_v at mammalian physiological temperatures. Any residual effects at physiological temperatures would enable a persistent nervous system perturbation that might induce long term functional changes in nociceptor function.

4.2. The Molecular Basis of The Cellular Actions of Permethrin on Nociceptors. In our acute treatment studies, we described a wide range of potent interactions between permethrin and the peripheral pain system. We also obtained evidence of unique cellular consequences that were limited to certain deep tissue nociceptors. Most cellular and molecular affects were uniformly manifested in both superficial and deep nociceptor populations. These included: 1) A TTXr $Na_v1.8$ dependent increase in AP duration; 2) Elimination of the AHP phase of the AP; 3) A >10 mV hyperpolarization of the $V_{1/2}$ for $Na_v1.8$ activation; 4) A two fold increase in the tau_{deact} of $Na_v1.8$; and 5) A nearly 3 fold increase in the tau_{inact} of $Na_v1.8$. Both the range and degree of these interactions were impressive and certainly much greater than would accompany exposure to an endogenous pro-inflammatory mediator associated with the development of allodynia (e.g., England et al., 1996; Gold et al., 1996; Gold et al., 1998).

Despite broadly similar molecular influences of permethrin, functional cellular differences were observed between superficial and deep tissue nociceptors. These differences were manifested as the production of TTXr $Na_v1.8$ dependent excitability shifts and spontaneous activity that were only exhibited by muscle nociceptors. Attempts to identify the molecular basis of the specific functional influences on muscle nociceptors were unsuccessful. Regardless of whether nociceptor classes exhibited spontaneous activity, the pyrethroid-induced changes in $Na_v1.8$ activation, deactivation, and inactivation were uniform across nociceptor subgroups, and could not account for changes cellular reactivity to permethrin.

With this in mind, we specifically probed the involvement of another family of ion channels, K_v7 (KCNQ), that are known to be important in the generation of spontaneous activity (Marrion,

1997; Robbins, 2001), and that exhibit a selective distribution in DRG (Passmore et al., 2003; Linely et al., 2008). Consistent with this hypothesis, we could not identify K_v7 mediated currents in type 2 skin nociceptors. These nociceptors had failed to exhibit any spontaneous discharge or excitability shifts following permethrin exposure. However both type 8 and type 5 neurons did express a linopirdine sensitive K_v7 current ($K_v7.3$), but only type 5 muscle neurons became spontaneous. In any case, we were unable to detect any direct effects of permethrin on voltage dependent K_v7 currents in muscle nociceptors that could have contributed to spontaneous activity or excitability. While pyrethroids exert influences on other neuronal ion channels that are present in nociceptors (Soderlund et al., 2002; Bradberry et al., 2005; Burr and Ray, 2006; Ray and Fry, 2006; Clark and Symington, 2011) it is not likely these would contribute to the acute activation of these neurons.

As TTX was unable to reduce permethrin-induced spontaneous discharges or excitability shifts in muscle nociceptors (figure 2), the activity was necessarily dependent upon permethrin modified TTXr channel proteins, such as $Na_v1.8$, rather than the many other mammalian TTXs proteins that are modified by pyrethroids (including $Na_v1.2$, $Na_v1.3$ and $Na_v1.6$; Song and Narahashi, 1996b; Song et al., 1996; Smith and Soderlund, 1998; Meacham et al., 2008; Tan and Soderlund, 2009; Tan and Soderlund, 2010; McCavera and Soderlund, 2012). Nociceptors also widely express the TTXr $Na_v1.9$ (Fang et al., 2002; Jiang and Cooper, 2011). Given the known effectiveness of pyrethroids on TTXr Na_v, a differential role for $Na_v1.9$ in muscle nociceptors reactivity is a possibility. Unlike all other known Na_v, this slowly activating and ultraslow inactivating ion channel protein does not participate in action potential formation (Cummins et al., 1999; Dib-Hajj et al., 2002), but it may contribute to initiation and bursting discharge (Maingret et al., 2008). It is not known whether pyrethroids have any influence on the physiology of $Na_v1.9$. This unusual Na_v has been identified in several sub-classified nociceptor groups, including type 2 skin and type 8 vascular nociceptors that were examined in this report (Jiang and Cooper, 2011; Rau et al., 2012); however we did not observe any spontaneous activity or excitability shifts in those nociceptor groups. It is possible that those factors that render muscle nociceptors uniquely vulnerable to permethrin are more quantitative than qualitative. Perhaps the balance between ionic fluxes that initiate (e.g., $Na_v1.7$, $Na_v1.8$) or oppose action potential formation (e.g., K_v7, K_{ca}, K_A) are more important than the presence and permethrin vulnerability of any given ion channel protein.

4.3. Residual Influences of Permethrin at Physiological Body Temperature. Humans are highly resistant to pyrethroid poisoning. A number of factors (absorption, elimination, innate sensitivity, body temperature) contribute to the lower lethality of pyrethroids in humans (Ray and Frey, 2006; Narahashi et al., 2007). At ~20° C, pyrethroids exert profound effects on Na_v proteins that are fatal to insects; but at mammalian physiological temperatures, some of these influences are greatly reduced (i.e., deactivation; Song and Narahashi, 1996b; see also Motomura and Narahashi, 2000). Given that humans report pain and paresthesia following ingestion or superficial contact with pyrethroids makes it likely that significant, sub-lethal, modulation of Na_v physiology is possible in humans. Consistent with this, we were able to demonstrate that physiological temperatures only reversed a portion of permethrin's influences. Across the full range of modulatory effects, significant cellular (AP duration, AHP, spontaneous activity) and molecular effects (tau_{deact}, activation $V_{1/2}$ of $Na_v1.8$) were retained at 35° C. With this pattern of outcomes, the pain described by humans is more readily understood.

The mechanism of the partial recovery of Na_v activity is obscure, as there is little evidence specifically for TTXr $Na_v1.8$. Regardless, the temperature dependent effects could be understood as an increased rate of permethrin dissociation or state dependent transitions (see Chin and Narahashi, 1986; Vais et al., 2000). We must also consider the much faster kinetics and conductance exhibited by Na_v at 35° C (3 fold slower at room temperature by some measures; Rosen, 2001; see also Milburn et al., 1995). The deactivation and inactivation time constants are certainly influenced greatly by ambient temperature independent of the presence of pyrethroids. Some portion of the normalization of Na_v kinetics, in the presence of permethrin, is attributable to the sensitivity of this channel protein to ambient temperature. Distinct thermal influences on various aspects of pyrethroid -Na_v physiology appear likely as the shifts we observed in the activation $V_{.50}$ cannot be partially explained by thermal kinetics. The voltage dependence of Na_v activation is not influenced by ambient temperature (TTX sensitive Na_v; Rosen, 2001). Yet we have shown that the activation $V_{.50}$ recovers significantly from permethrin modulation at 35° C.

As many of the influences of permethrin are retained in $Na_v1.8$ near physiological temperatures, there is reason to suspect that chronic exposure to permethrin, at sufficient concentrations, could perturb human nociceptor function in a significant manner in conditions in which nociceptors are activated or even when they are functionally quiescent. Although nociceptors rarely discharge action potentials spontaneously, a certain degree of single channel activity is always present. In DRG neurons, tetramethrin modified single channel open time and duration to a much greater extent in TTXr $Na_v1.8$ than in TTXs channels (Song and Narahashi, 1996a). The heightened influx of Na^{++}, in neurons expressing $Na_v1.8$ (nociceptors), could set into motion compensatory mechanisms that have been shown to modify neuronal excitability or produce spontaneous activity (Grubb and Burrone, 2010; Kuba et al., 2010). The mechanisms underlying 'activity dependent plasticity' are not known (Clark et al., 2009), but permethrin can also alter the expression of intracellular proteins that can influence neuronal excitability (Harrill et al., 2006; Harrill et al., 2010).

We have demonstrated that permethrin can alter nociceptor excitability at physiological temperatures. This phenomenon may be informative with respect to the potential role of permethrin in the chronic pain associated with Gulf War Illness (GWI). Many soldiers returning from the Persian Gulf War reported chronic deep tissue pains that were not associated with any traumatic event (Haley et al., 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006). The Research Advisory Committee on Gulf War Illness determined that pesticides may have contributed to the development of the symptoms of GWI (Binns et al., 2008). During the brief course of the Gulf War, veterans were potentially exposed to 67 insecticides and repellants containing 37 distinct ingredients (DOD Environmental Exposure Report: Pesticides, 2003; see Binns et al., 2008). Due to its interaction with key mammalian peripheral nervous system proteins, the most significant insecticide may have been permethrin. While deployed, soldiers were provided permethrin to apply to their uniforms. Reports suggest that the prescribed application procedures may not have been followed closely. As a result, the daily exposure levels could have been high, and the physiological impacts of this contact could have been compounded by simultaneous exposures to sarin gas, depleted uranium, adjuvants, nerve gas prophylactics (i.e., pyridostigmine bromide) as well as a variety of other insecticides and repellants (Binns et al., 2008; Brimfield, 2012; Israel, 2012). Over a period of months of exposure, the consequences of these perturbations might result in significant

molecular maladaptations to Na_v or other membrane proteins expressed in the affected afferent neuron pool.

In conclusion, the type I pyrethroid permethrin exerts profound cellular and molecular effects on nociceptors via its interaction with $Na_v1.8$. While the influence of permethrin is mainly uniform across nociceptor populations innervating superficial and deep tissue, permethrin especially impacted functional activity in one population of muscle nociceptors. Although all effects were diminished at mammalian physiological temperatures, a significant proportion of the molecular and cellular influences were retained at 35° C. Accordingly, prolonged exposure to permethrin could constitute a significant disturbance to nociceptive neuron physiology and thereby set into motion certain direct and indirect adaptations with adverse consequences.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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The funding agency had no role in the collection, analysis, or interpretation of the data. They did not participate in the writing of the report nor in the decision to submit the paper for publication.

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Figure Captions

Figure 1. Permethrin Increases AP Duration in Nociceptors. A-C) Action potentials in tissue specific nociceptors following exposure to ETOH vehicle. The arrow highlights the AHP. **D-F)** Action potentials in the same nociceptors following exposure to 10 μM permethrin for 6-10 minutes. After-hyperpolarizations are absent in all treated neurons. **G)** Summary data for 3 nociceptive classes. Duration increases were statistically significant ($p<.001$; paired T-test). Action potentials were evoked by a 3 nA injection for 1 ms.

Figure 2. Permethrin Increases Excitability and Induces Spontaneous Activity in Muscle Nociceptors. A) Excitability testing in a type 5 muscle nociceptor. Ten consecutive current injection tests in an ETOH (upper panel) and Permethrin treated case (lower panel; 10 μM, 10 min). Vertical green bars separate consecutive tests. Substantial increases in action potential discharge were evident. **B)** Summary data for excitability testing indicated that only type 5 muscle nociceptors manifested excitability shifts. TTX did not block shifts in AP excitability (** $p<.03$ vs ETOH). **C)** Representative spontaneous activity in a type 5 muscle nociceptor after exposure to permethrin (upper trace; 10 μM). Five minutes of observation are shown. Scale bars: 100 mV and 30 sec; In the lower panel a rare burst discharge from a type 5 nociceptor exposed to permethrin; Scale bars 80 mV and 150 msec. **D)** Summary data of the influence of permethrin on spontaneous activity. Only type 5 muscle nociceptors exhibited spontaneous activity. TTX did not prevent the development of spontaneous activity. * $p<.03$ vs ETOH (Mann-Whitney U test; ties were not corrected). *** $p<.001$

Figure 3. Permethrin Shifted the Voltage Dependent Activation of $Na_v1.8$. Hyperpolarizing shifts in the activation $V_{1/2}$ suggested greater excitability following permethrin exposure (10 μM): **A)** Skin nociceptors; **B)** Muscle nociceptors; Representative traces are presented as inserts. Upper trace: pre-permethrin; lower trace: post permethrin (voltage step to 0 mV); **C)** Vascular

nociceptors. Representative traces are presented as inserts. Upper trace: pre-permethrin; lower trace: post-permethrin (voltage step to 0 mV). Scale bars in 'B' apply.

Figure 4. Permethrin Shifts Voltage Dependence of Deactivation in All Tissue Specific Nociceptors. A-C) ETOH exposed cells exhibit a relatively consistent pattern of deactivation across membrane voltages (black curves). Following treatment with permethrin, deactivation time constants were increased: (skin: F=22.1, DF=1.9; muscle: F=10.2, DF=1,11; vascular: F=28.4, DF=1,14) and a strong voltage dependence appeared (red curves). The interaction between voltage and time constants were statistically significant for all tissue nociceptors (skin: F=6.04, p<.0001; muscle: F=3.49, p<.005; vascular: F=5.49, p<.0001). There were no statistical differences between tissue specific nociceptors. **D-F)** Representative traces illustrate the deactivation tail current associated with permethrin and ETOH treated neurons. All traces taken from a single deactivation voltage step to -40 mV. The arrow signifies the point at which the voltage is abruptly clamped at -40 mV. **G)** A schematic of the deactivation protocol. The voltage step to 0 mV was 8 ms long. The deactivation voltage steps were 150 ms. 10 μM permethrin applied for 2 minutes.

Figure 5. Permethrin and Fast Inactivation of $Na_v1.8$. A-C) Voltage dependent inactivation of $Na_v1.8$ was not significantly modified by application of 10 μM permethrin (2 minutes); The tau_{inact} (ms) was significantly increased in all cases. **D)** A schematic of the inactivation protocol. Inserts show representative traces (upper: ETOH; lower: Permethrin). Note the slowing of inactivation after permethrin. The cells were pre-pulsed in 5 mV steps from -10 to -80 mV. The test pulse was 0 mV, 150 ms.

Figure 6. Permethrin did not Modify Currents Passing Through K_v7 Proteins. A) The voltage dependence of K_v7 is similar after treatment with permethrin (4 minutes; type 5 muscle nociceptors). Inset: A schematic representation of the voltage protocol used to evoke K_v7 currents. **B)** Immunocytochemistry conducted on type 5 and type 8 cells demonstrates positive

staining for $K_v7.3$ (right panel). Bright field images are presented in the left panel. **C)** Summary data of the averaged peak amplitude of permethrin and ETOH treated K_v7 proteins expressed in type 2 (skin), type 5 (muscle) and type 8 (vascular) nociceptors. Skin nociceptors did not express a linopirdine sensitive current. **D)** Representative traces of linopirdine sensitive current (subtraction currents in the presence of ETOH or permethrin) evoked at -40 mV. The arrows indicate the linopirdine sensitive 'tail' currents.

Figure 7. Thermal Modulation of Permethrin Influences on Action Potential Duration and Spontaneous Activity. A) Permethrin-induced increases in action potential duration were diminished at 35° C. Action potential duration remained significantly increased relative to ETOH treated neurons. ** significantly different from the ETOH test ($p<.05$; Wilcoxen); * significantly different from ETOH and Permethrin tests at 21° C ($p<.05$; Wilcoxen). **B)** Representative action potentials of a type 5 muscle nociceptor at the three conditions. The AHP exhibits a partial recovery (arrow; -16.2 +/- 1.2 vs -5.2 +/- 2.3 mV). **C)** Representative action potentials of a type 8 nociceptor. Note that the AHPs recovered poorly (arrow; -14.4 +/- 1.3 vs -6.0 +/- 1.7 mV). **D)** Representative action potentials of a type 2 skin nociceptor at the three conditions. The AHP exhibits partial recovery at 35° C (arrow; -18.8 +/-1.1 vs -7.1 +/- 0.26 mV). **E)** Physiological temperatures significantly reduced the spontaneous action potentials evoked by permethrin (5 min observation; muscle nociceptors).

Figure 8. Permethrin Influences on $Na_v1.8$ are Retained at 35° C. **A)** Summary data for $Na_v1.8$ deactivation. Under the influence of permethrin, the tau_{deact} was significantly increased at 35° C relative to ETOH treated cases ($p<.0004$), but time constants remained significantly shorter than those at 21° C ($p<.0006$). **B-D)** Representative $Na_v1.8$ currents evoked by a voltage command step to 0 mV, and deactivated to -70 mV. In 'B', the deactivation tail current is barely visible at 35° C. Following permethrin presentation, a large tail current is obvious at 35° C. When the temperature is changed to 21° C, the tail current is greatly increased. All traces represent the same neuron. **E)** Although the effects on $V_{1/2}$ were reduced, permethrin shifted

voltage dependent activation substantially at 35° C. **F-H)** Representative $Na_v1.8$ currents evoked by a voltage command step to -10 mV. All traces represent the same neuron.

Figures

Figure 1

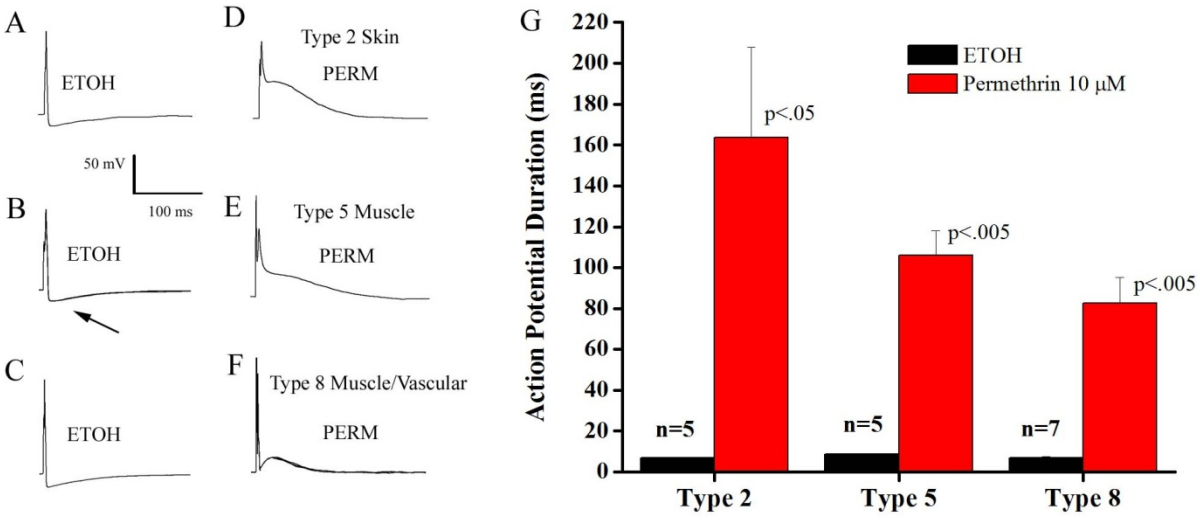

A
ETOH
D
Type 2 Skin
PERM
50 mV
100 ms
B
ETOH
E
Type 5 Muscle
PERM
C
ETOH
F
Type 8 Muscle/Vascular
PERM
G
Action Potential Duration (ms)
220
200
180
160
140
120
100
80
60
40
20
0
ETOH
Permethrin 10 µM
p<.05
p<.005
p<.005
n=5
n=5
n=7
Type 2
Type 5
Type 8

Figure 2

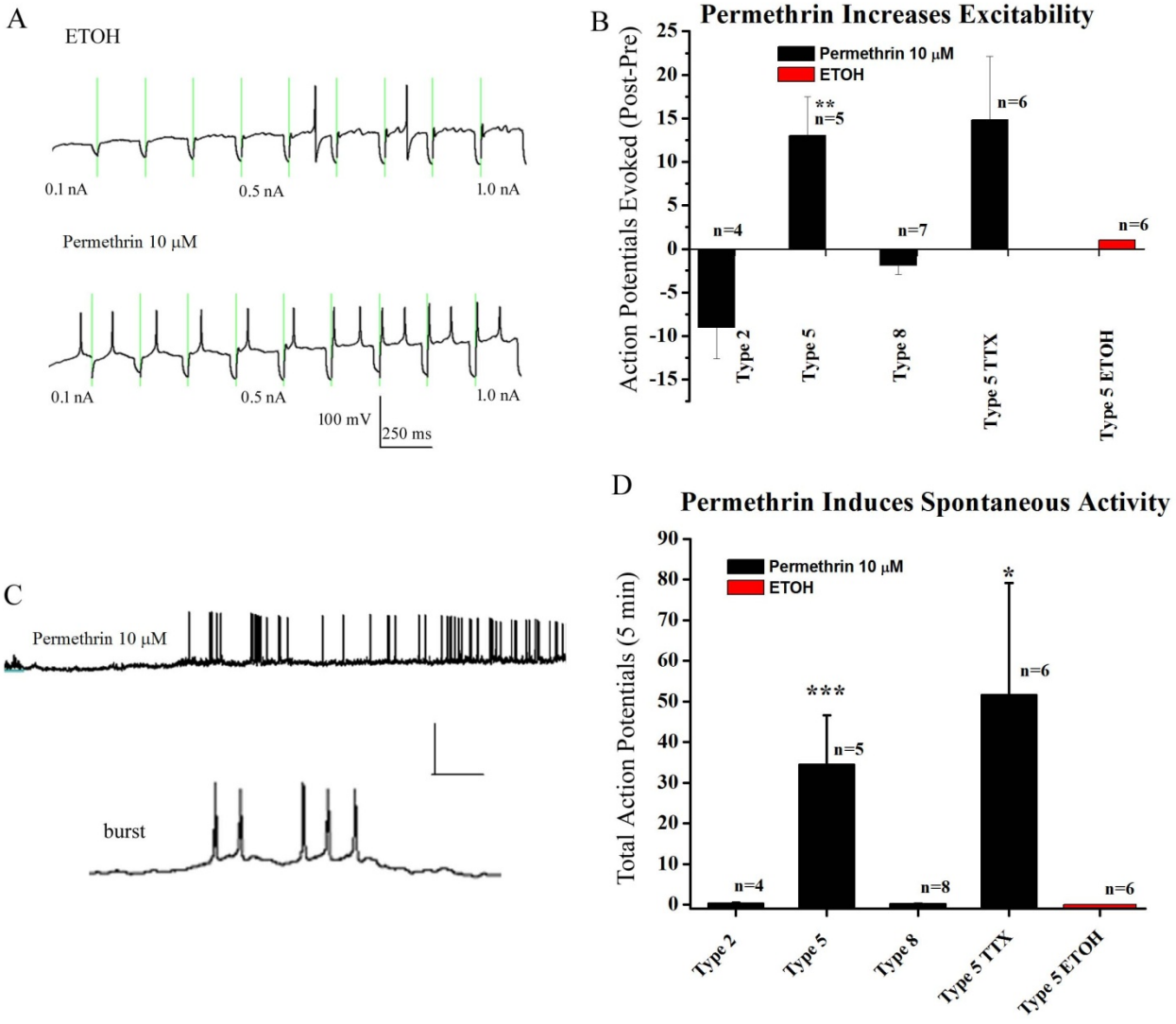

A
ETOH
0.1 nA
0.5 nA
1.0 nA
Permethrin 10 µM
0.1 nA
0.5 nA
1.0 nA
100 mV
250 ms
B
Permethrin Increases Excitability
Permethrin 10 µM
ETOH
Action Potentials Evoked (Post-Pre)
**
n=5
n=6
n=4
n=7
n=6
Type 2
Type 5
Type 8
Type 5 TTX
Type 5 ETOH
C
Permethrin 10 µM
burst
D
Permethrin Induces Spontaneous Activity
Permethrin 10 µM
ETOH
Total Action Potentials (5 min)
***
n=5
*
n=6
n=4
n=8
n=6
Type 2
Type 5
Type 8
Type 5 TTX
Type 5 ETOH

Figure 3

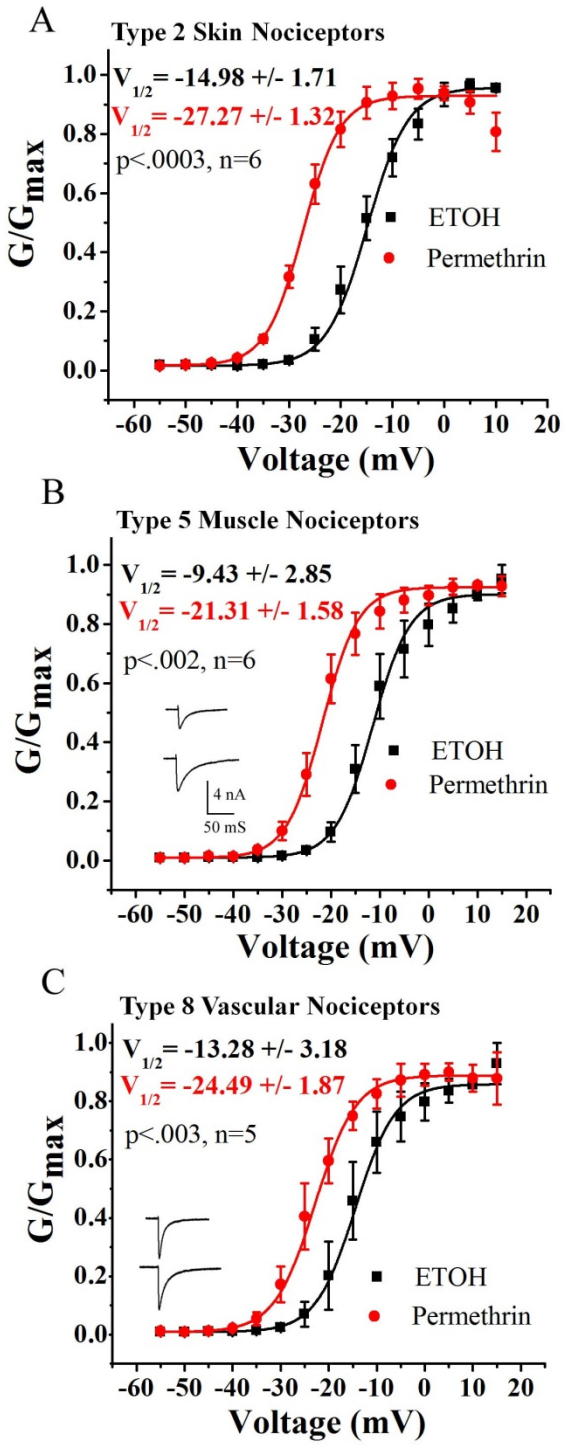

A
Type 2 Skin Nociceptors
V1/2= -14.98 +/- 1.71
V1/2= -27.27 +/- 1.32
p<.0003, n=6
ETOH
Permethrin
G/Gmax
Voltage (mV)
B
Type 5 Muscle Nociceptors
V1/2= -9.43 +/- 2.85
V1/2= -21.31 +/- 1.58
p<.002, n=6
4 nA
50 mS
ETOH
Permethrin
G/Gmax
Voltage (mV)
C
Type 8 Vascular Nociceptors
V1/2= -13.28 +/- 3.18
V1/2= -24.49 +/- 1.87
p<.003, n=5
ETOH
Permethrin
G/Gmax
Voltage (mV)

Figure 4

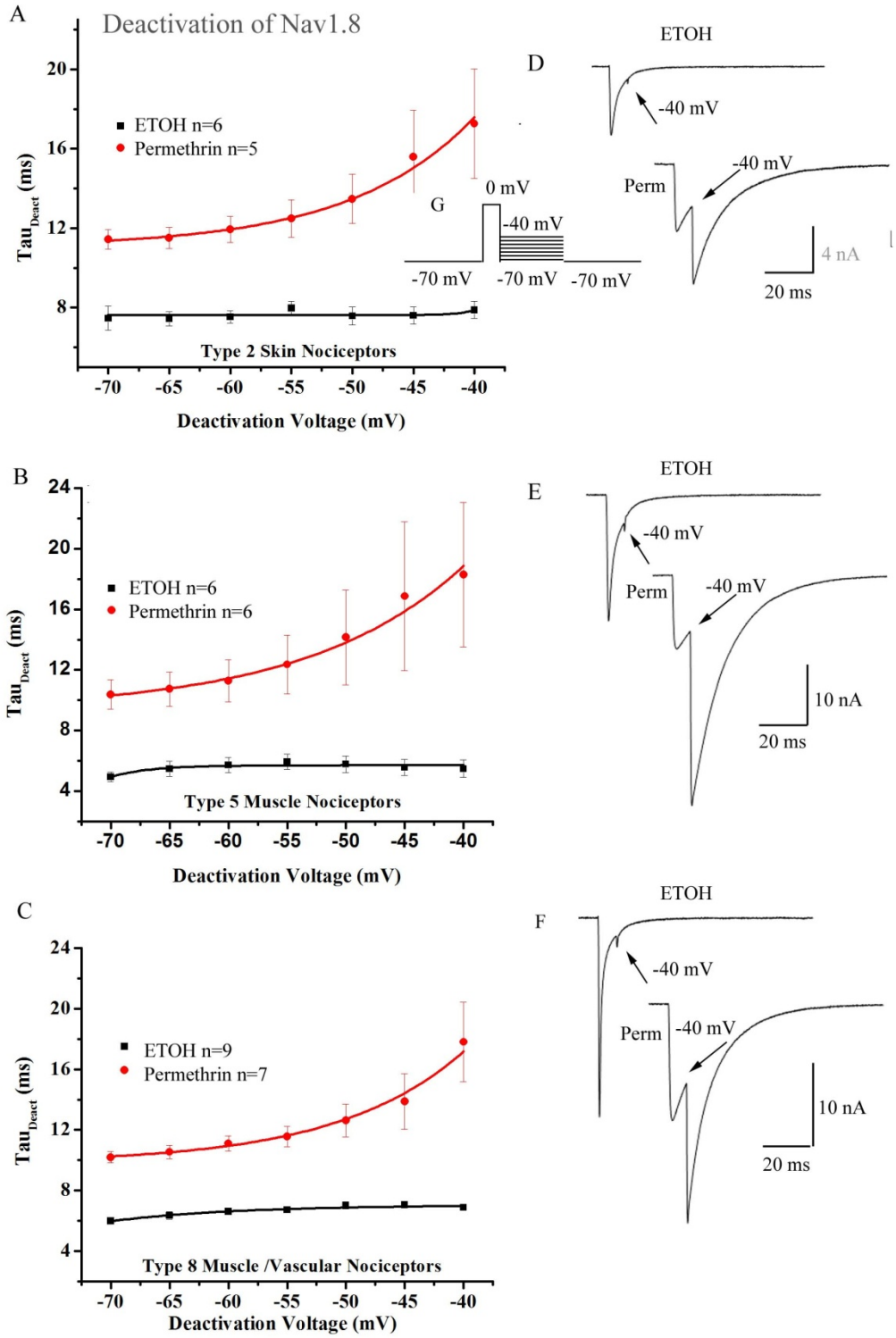

A
Deactivation of Nav1.8
ETOH n=6
Permethrin n=5
Tau Deact (ms)
Type 2 Skin Nociceptors
Deactivation Voltage (mV)
G
0 mV
-40 mV
-70 mV
-70 mV
-70 mV
D
ETOH
-40 mV
Perm
-40 mV
4 nA
20 ms
B
ETOH n=6
Permethrin n=6
Tau Deact (ms)
Type 5 Muscle Nociceptors
Deactivation Voltage (mV)
E
ETOH
-40 mV
Perm
-40 mV
10 nA
20 ms
C
ETOH n=9
Permethrin n=7
Tau Deact (ms)
Type 8 Muscle /Vascular Nociceptors
Deactivation Voltage (mV)
F
ETOH
-40 mV
Perm
-40 mV
10 nA
20 ms

Figure 5

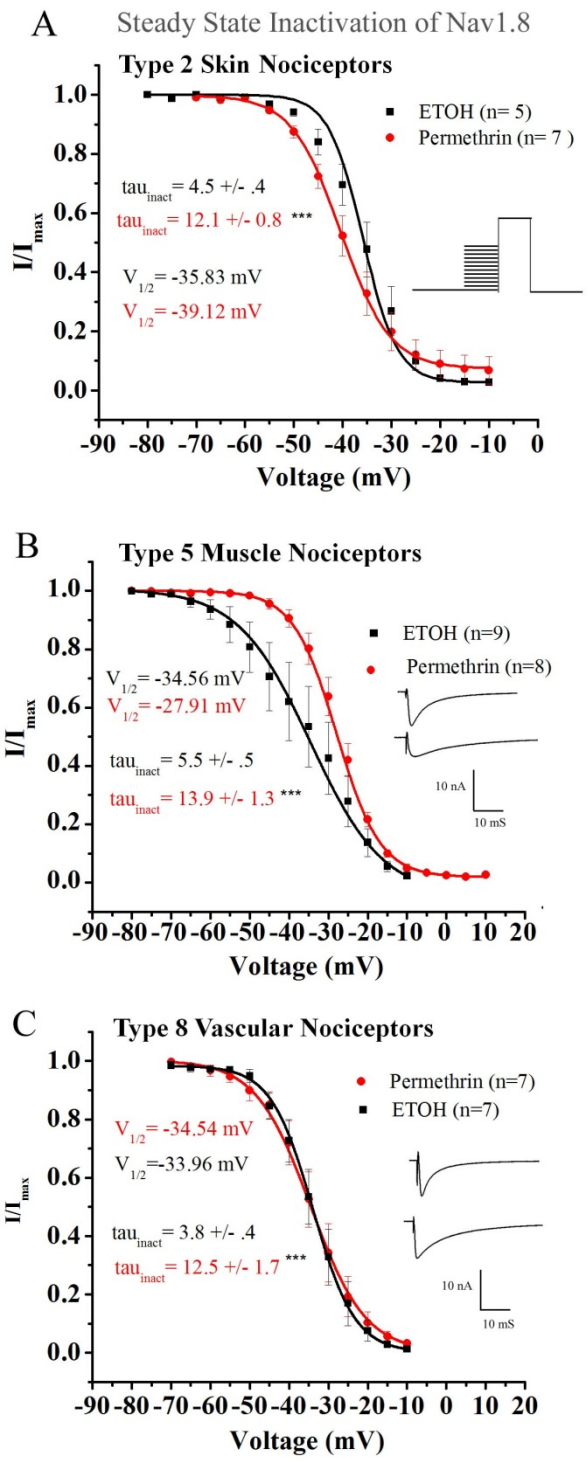

A
Steady State Inactivation of Nav1.8
Type 2 Skin Nociceptors
ETOH (n= 5)
Permethrin (n= 7)
tau_inact = 4.5 +/- .4
tau_inact = 12.1 +/- 0.8 ***
V1/2 = -35.83 mV
V1/2 = -39.12 mV
I/Imax
Voltage (mV)
B
Type 5 Muscle Nociceptors
ETOH (n=9)
Permethrin (n=8)
V1/2 = -34.56 mV
V1/2 = -27.91 mV
tau_inact = 5.5 +/- .5
tau_inact = 13.9 +/- 1.3 ***
10 nA
10 mS
I/Imax
Voltage (mV)
C
Type 8 Vascular Nociceptors
Permethrin (n=7)
ETOH (n=7)
V1/2 = -34.54 mV
V1/2 =-33.96 mV
tau_inact = 3.8 +/- .4
tau_inact = 12.5 +/- 1.7 ***
10 nA
10 mS
I/Imax
Voltage (mV)

Figure 6

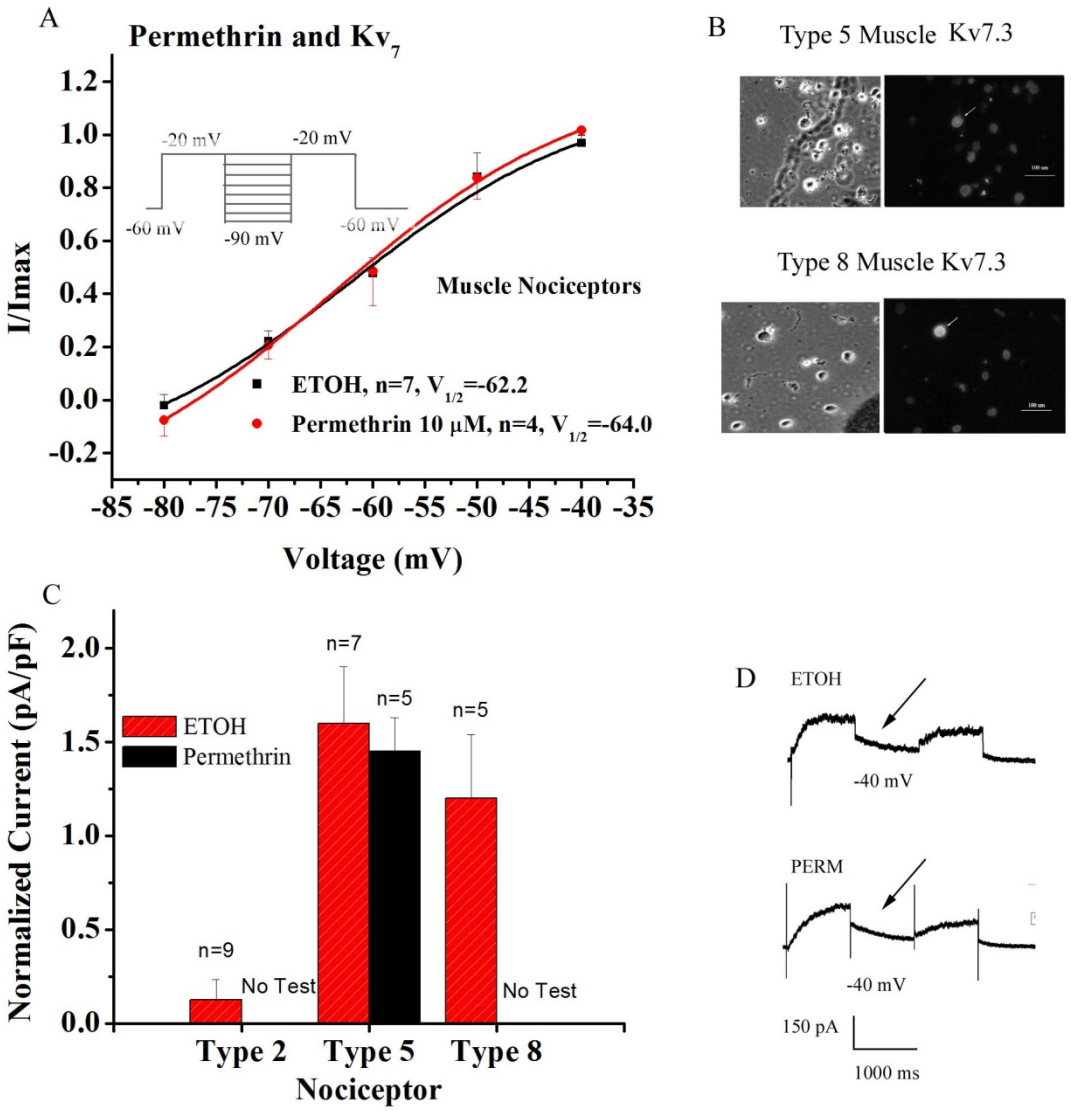

A
Permethrin and Kv7
I/Imax
Voltage (mV)
-20 mV
-20 mV
-60 mV
-90 mV
-60 mV
Muscle Nociceptors
ETOH, n=7, V1/2=-62.2
Permethrin 10 µM, n=4, V1/2=-64.0
B
Type 5 Muscle Kv7.3
Type 8 Muscle Kv7.3
C
Normalized Current (pA/pF)
ETOH
Permethrin
n=9
No Test
n=7
n=5
n=5
No Test
Type 2
Type 5
Type 8
Nociceptor
D
ETOH
-40 mV
PERM
-40 mV
150 pA
1000 ms

Figure 7

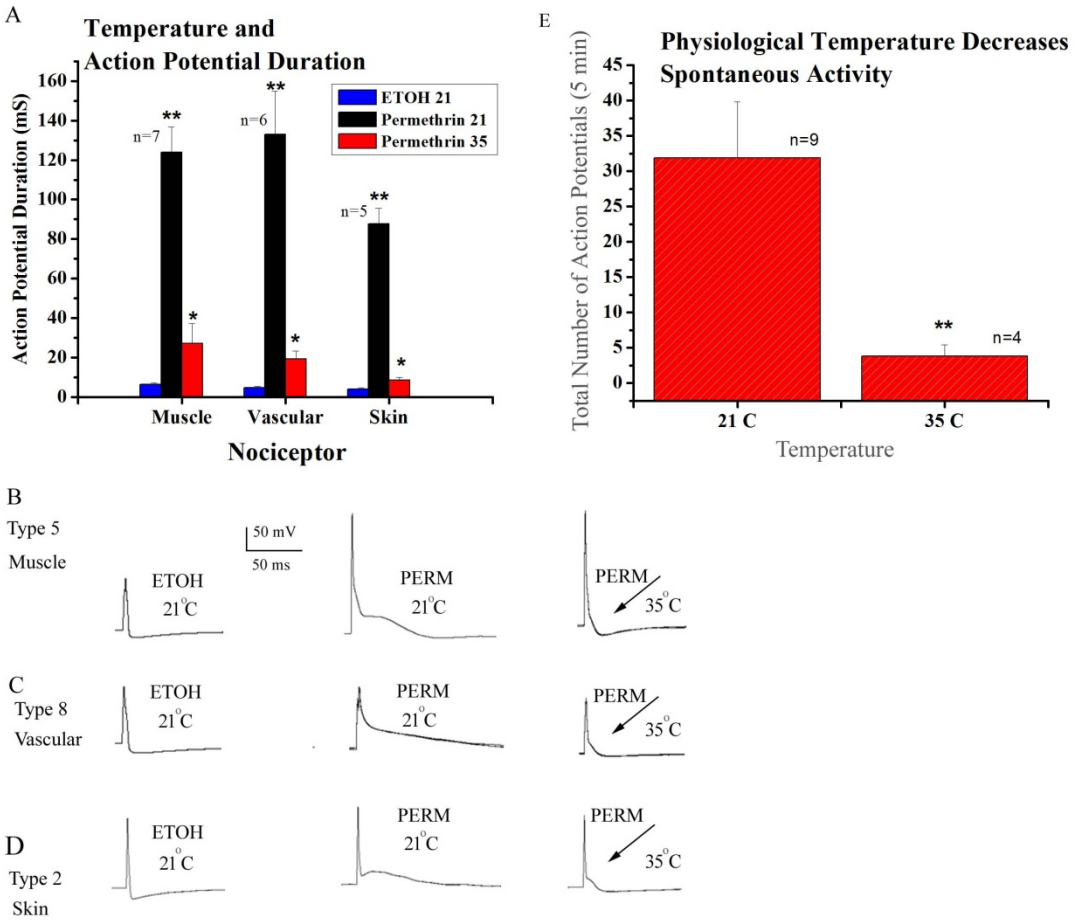
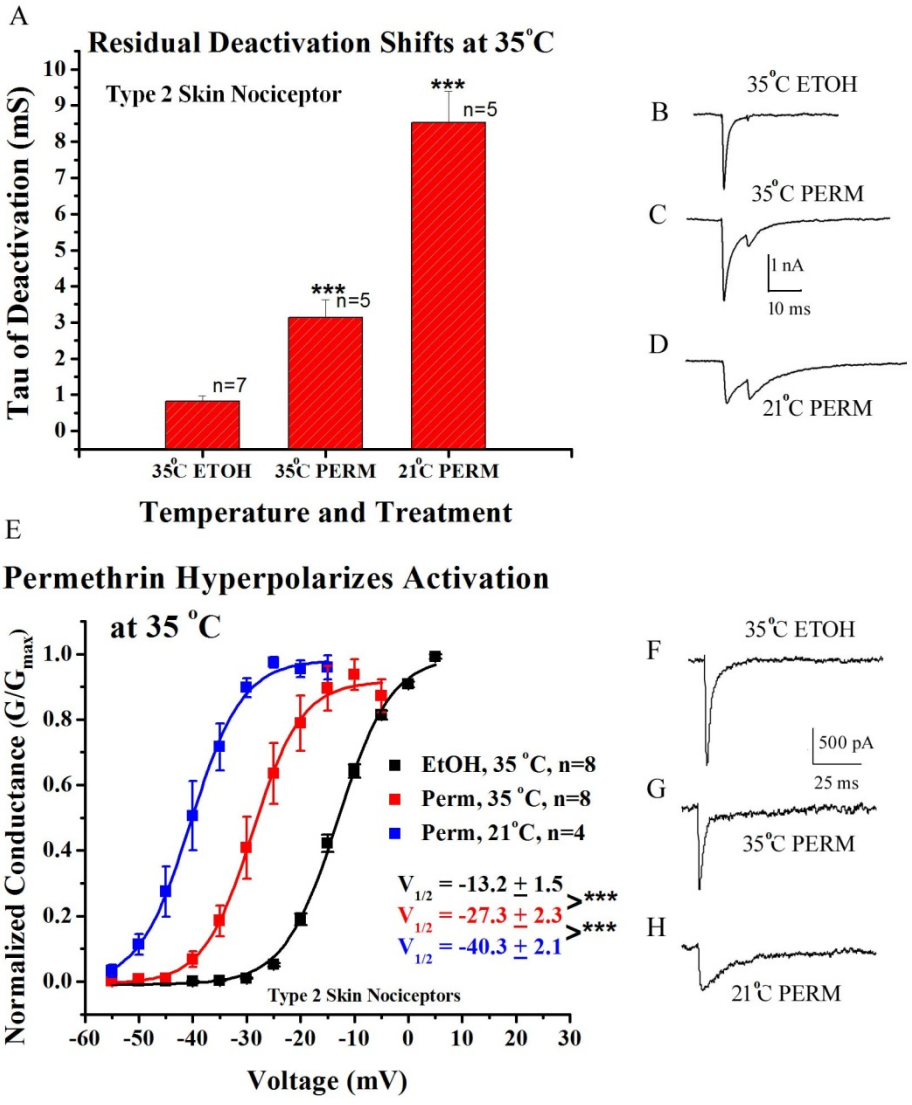

A
Temperature and
Action Potential Duration
Action Potential Duration (mS)
ETOH 21
Permethrin 21
Permethrin 35
n=7
n=6
n=5
Muscle
Vascular
Skin
Nociceptor
E
Physiological Temperature Decreases
Spontaneous Activity
Total Number of Action Potentials (5 min)
n=9
n=4
21 C
35 C
Temperature
B
Type 5
Muscle
50 mV
50 ms
ETOH
21°C
PERM
21°C
PERM
35°C
C
Type 8
Vascular
D
Type 2
Skin

Figure 8


A
Residual Deactivation Shifts at 35°C
Type 2 Skin Nociceptor
Tau of Deactivation (mS)
***
n=5
***
n=5
n=7
35°C ETOH
35°C PERM
21°C PERM
Temperature and Treatment
B
35°C ETOH
C
35°C PERM
1 nA
10 ms
D
21°C PERM
E
Permethrin Hyperpolarizes Activation
at 35 °C
Normalized Conductance (G/Gmax)
EtOH, 35 °C, n=8
Perm, 35 °C, n=8
Perm, 21°C, n=4
V1/2 = -13.2 ± 1.5
V1/2 = -27.3 ± 2.3 >***
V1/2 = -40.3 ± 2.1 >***
Type 2 Skin Nociceptors
Voltage (mV)
F
35°C ETOH
500 pA
25 ms
G
35°C PERM
H
21°C PERM

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Persistent Na^+ and K^+ Channel Dysfunctions after Chronic Exposure to Insecticides and Pyridostigmine Bromide

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Abstract

Many soldiers that served in the 1991 Gulf War developed widespread chronic pain. Exposure to insecticides and the nerve gas prophylactic pyridostigmine bromide (PB) were identified as risk factors by the Research Advisory Committee on Gulf War Veterans' Illnesses (GWI). We examined whether a 60 day exposure to neurotoxicants/PB (NTPB) produced behavioral molecular and cellular indices of chronic pain in the rat. Male rats were exposed to chlorpyrifos (120 mg/kg; SC), permethrin (2.6 mg/kg; topical), and PB (13.0 mg/kg; oral) or their respective vehicles (corn oil, ethanol, water). Permethrin can exert profound influences on voltage activated Na^+ channel proteins; while chlorpyrifos and PB can increase absorption and/or retard metabolism of permethrin as well as inhibit cholinesterases. During and after exposure to these agents, we assessed muscle pressure pain thresholds and activity (distance; rest time). Eight and 12 weeks after treatments ceased, we used whole cell patch electrophysiology to examine the physiology of tissue specific DRG nociceptor channel proteins expressed in muscle and putative vascular nociceptors (voltage dependent, activation, inactivation, deactivation). Behavioral indices were unchanged after treatment with NTPB. Eight weeks after treatments ended, the peak and average conductance of K_v7 mediated K^+ currents were significantly increased in vascular nociceptors. When a specific K_v7 inhibitor was applied (linopirdine, 10 μM), NTPB treated vascular nociceptors emitted significantly more spontaneous APs than vehicle treated neurons. Changes to K^+ channel physiology were resolved 12 weeks after treatment. The molecular alterations of Na^+ and K^+ channel proteins and the specific susceptibility of the vascular nociceptor population could be important for the etiology of GWI pain.

Keywords: Pain; Neurotoxicant; Nociceptor; Gulf War; $Na_v1.8$; K_v7

1.1 Introduction

Although lingering musculoskeletal pain is not uncommon among veterans, there are distinct multi-symptom manifestations of a widespread chronic pain associated with soldiers of the 1991 Gulf War. Despite the relative brevity of both the conflict and the deployment to the Persian Gulf region, there have been many published reports of unusual complexes of headache, joint pain, muscle pain, and abdominal pain in Gulf War veterans (GWV; Escalante and Fischbach, 1998; Fukuda et al., 1998; Proctor et al., 1998; Gray et al., 1999; Kang et al., 2000; Steele et al., 2000; Gray et al., 2002; Voelker et al 2002; Ang et al., 2006). When these, and other reports, were subjected to meta-analysis, it was confirmed that those who served in the Persian Gulf theatre were more likely to suffer from a variety chronic pain conditions. In particular, the relative likelihood of joint, muscle, and/or abdominal pain was found to be 3 fold greater in GWV than in those warfighters deployed elsewhere (Thomas et al., 2006; see also Kang et al., 2000; Kelsall et al., 2004; Stimpson et al 2006). These chronic pain conditions and other sensory, motor and cognitive disturbances are collectively recognized as Gulf War Illness (GWI).

A myriad of factors, including depleted uranium, sarin gas, oil fires, vaccination adjuvants, organophosphates, carbamates, stress and many others have been proposed and/or examined as potentially determinant or contributory factors in the development of GWI syndromes (Binns et al., 2008; see also Shoenfeld and Agmon-Levin, 2010). Substantial challenges remain before a specific etiology and effective treatment can be identified for this condition. To date, there has been relatively little examination of the instigating factors underlying the chronic pain component of GWI. The causal factors must be dissimilar to other chronic pain conditions. Although joint pain is a frequent complaint, there is no evidence of histological changes in joints, nor is there the presence of inflammatory markers that are associated with classic joint diseases such as osteo- or rheumatoid arthritis (Diaz-Torne et al 2007; Pessler et al., 2008). Although sensory disturbances may be present, there is no evidence of significant physical nerve impairment. Changes in conduction velocity, that can be indicative of segmental deymelination,

have not been observed in GWV (Bourdette et al., 2001; Sharief et al., 2002; Blanchard et al., 2006; but see Rivera-Zayas et al., 2001).

The Research Advisory Committee on Gulf War Illness determined that pesticides may have contributed to the development of the symptoms of GWI (Binns et al., 2008). Pesticides and repellants were used liberally in theatre. These included a variety of organophosphate, organochlorine, dialkylamide, carbamate and pyrethroid neurotoxicants. Some of these pesticides were prescribed for use at specific concentrations and with specific application methods and procedures. These application procedures were not always carefully followed and warfighters often supplemented prescribed agents with others they acquired on their own (US, DOD Environmental Exposure Report: Pesticides, 2003).

While stationed in the Gulf region, warfighters made ample use of the pyrethroid insecticides permethrin and allethrin (Binns et al., 2008). Burning, stinging and other skin sensations have been reported following either ingestion (Gotoh, et al., 1998) or topical contact with permethrin (Kolmodin-Hedman et al., 1982; Tucker and Flannigan, 1983; Flannigan and Tucker, 1985) or other pyrethroid formulations (Knox et al., 1984; Wilks, 2000; see Wolansky and Harrill, 2008). The influence of type I and type II pyrethroids (allethrin, tetramethrin, deltamethrin) on TTXs and TTXr Na_v voltage sensitive proteins of the DRG (dorsal root ganglion; $Na_v1.6$, $Na_v1.7$, Na_v 1.8) suggests a relatively direct pathway to nociceptor activation and pain (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996a,b; Tabarean and Narahashi, 1998; Tabarean and Narahashi 2001). Our recent investigations have documented a wide range of potent acute interactions of the type 1 pyrethroid, permethrin, with specifically identified skin, muscle and putative vascular nociceptor $Na_v1.8$ protein (Jiang et al., 2013). The findings included the generation of spontaneous activity in muscle, but not skin or vascular nociceptors. It is not clear that the acute influences of permethrin on nociceptor function will be manifested after chronic exposure to this neurotoxicant.

It is possible that synergisms between pyrethroid neurotoxicants and other agents that either promote their activity of otherwise interact with the pain system could result in significant structural or molecular disruptions with potential clinical import. Animal studies have revealed that various combinations of permethrin, chlorpyrifos, the nerve gas prophylactic pyridostigmine bromide and other anti-cholinesterases can produce a variety of motor and cognitive signs

(Abou-Donia et al., 2001; Abdel-Rahman et 2004; Abou-Donia et al 2004; Abdullah et al., 2011), suppress enzyme activity (Abdel-Rahman et al., 2002a; Abdel-Rahman et al 2004b; Abdullah et al., 2011) and degrade the blood brain barrier (Abdel-Rahman et al 2002; Abdel-Rahman et al., 2004b) at levels greater than can be produced by individual agents (but see Jortner, 2006; Wille et al., 2011); but the involvement of any pain system components are unreported. Permethrin, in particular, has close linkages to the pain system. The capacity of pyrethroids to directly interact with pain system ion channel proteins that are critical to excitability provides a natural focus for investigations of the deleterious effects of chronic neurotoxicant exposure.

In the studies described below, we examined how a 30 day exposure to permethrin, chlorpyrifos, and pyridostigmine bromide affected behavioral, cellular and molecular indices of pain in rodents. Molecular and cellular studies were targeted on discrete populations of skin, muscle and vascular nociceptors and those voltage sensitive ion channel proteins with substantial influence over nociceptor excitability ($Na_v1.8$; $K_v7.3$).

2.1 Methods

2.2 Preparation of Cells. Male Sprague-Dawley rats (n=60) were anesthetized (Isoflurane) and euthanized by decapitation. The spinal cord was dissected free and split longitudinally. The dorsal root ganglia were excised, trimmed, cut into several sections and digested at 35º C in a Tyrode's solution containing 2 mg/ml type 1 collagenase and 5 mg/ml Dispase II (90 min; Sigma-Aldrich; Roche Chemicals). Digested ganglia were dispersed by titruration and digested for an additional 45 minutes. Isolated neurons were spun down (1000 rpm), re-suspended and plated on 10, 35 mm Petri dishes. Plated neurons were bathed continuously in Tyrode's solution, containing (in mM): 140 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO (U.S. Army Medical Research and Materiel Command).

2.3 Recording and Characterization of Muscle and Vascular Nociceptors. Conventional whole cell patch techniques were used. Recordings were made with an Axopatch 200B or Multiclamp700 (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and a Digidata 1322A (Molecular Devices). Series resistance (R_s) was compensated 65-70% with Axopatch compensation circuitry. Whole cell resistance and capacitance was determined by the Clampex software utility from a capacitive trace. Recorded currents were sampled at 10-20 kHz and filtered at 2 kHz (Bessel filter). Electrodes (2-5 MΩ) were formed from borosilicate glass stock that was pulled to a suitable tip size by a Sutter P1000 (Sutter Instruments, Novato, CA). Recordings were completed within 2-10 h after plating. Only one cell was used per Petri dish. Studies on Na_v and K_v were conducted at room temperature. Some action potential studies were conducted at 35º C.

Cells were classified as muscle or vascular nociceptors according to the pattern of voltage activated currents evoked by three protocols (Cardenas et al., 1995; Petruska et al., 2002; Rau et al., 2007). All reported data was derived from recordings made on type 5 (C or group IV nociceptor) and type 8 (Aδ or group III nociceptor). Type 5 were identified as muscle nociceptors in prior tracing studies (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2012). Type 8 nociceptors were traced from all tissue sites (skin, muscle, colon, mucosa; Rau et al., 2007; Rau et al., 2012) and are presumed to be vascular in origin. Although the nociceptors we have tested in these studies are important classes of capsaicin (heat) sensitive nociceptors, additional classes of nociceptors can be traced from muscle, skin and other tissues (Rau et al., 2012).

2.4 Exposure Protocol. Juvenile male rats (90-110 g) were used in all studies. By the end of the experiments the average weights were 359+/-4 (vehicle) and 341+/-7 g (treated; $p<.03$). On arrival, rats were acclimated to the behavioral procedures for 2 weeks before dosing began. Neurotoxicants (NT) and PB were administered over a 60 day period. During this 60 day period, permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), in ETOH, was applied every day to a shaved area of the back. Chlorpyrifos (120 mg/kg; Sigma Aldrich) was administered by subcutaneous injection (corn oil) once every 14 days. PB was given by oral gavage (13 mg/kg; water) for 14 consecutive days, beginning at day 1. Animals were sacrificed for electrophysiological experiments 8 and 12 weeks after the dosing period had ended. Groups

of rats consisting of animals receiving equal volumes of ETOH, corn oil and water over the identical time period served as controls.

2.5 Spontaneous Activity Experiments. Following classification as a muscle or vascular nociceptor, cells were brought into current clamp mode. Cells were observed for spontaneous activity for 90 seconds. Subsequently the superfusion temperature was increased to 35º C (TC_{bip}; Cell Microsystems). Cells were reexamined for the presence of spontaneous activity over a 90 second observation period. In other experiments, linopirdine was applied for 6 minutes. The cell was brought into current clamp mode and action potential discharge, if present, were recorded for 1 minute. The total number of action potentials observed were counted off-line.

2.6 Isolation and Characterization of K_v7 Protein Currents. Voltage activated K^+ channels were characterized in a 'K-Iso' solution containing (in mM) 130 N-methyl-d-glucamine, 4 KCl, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-amino pyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with HCl. The reversal potential for K^+ was calculated to be -86 mV. The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

A current subtraction method was used to isolate K_v7 mediated currents from other K^+ currents that were present as tail currents. Following application of a K-Iso solution containing .001% ETOH (2 min application), the K_v7 voltage protocol was presented (see below). Subsequently, a K-Iso solution containing the K_v7 inhibitor, linopirdine (10 μM; Sigma Aldrich) was presented for 2 minutes. The K_v7 voltage protocol was repeated. Linopirdine treated currents were subtracted from the ETOH treated currents to obtain the linopirdine sensitive, K_v7, current. These data were collected 8 and 12 weeks after dosing had concluded from rats that had been neurotoxicant or vehicle treated for 60 days.

The amplitude of the K_v7 'tail' current was measured for 1000 ms from a point 10 ms after the repolarizing voltage step to the point 10 ms prior to the return step voltage. Because cell size varied substantially, the amplitude of the K_v7 linopirdine sensitive currents was normalized to cell capacitance (pA/pf). The normalized tail currents were averaged (from -30 mV to -70 mV) and the peak current was also identified. Tail currents (pA/pF) were normalized to the peak current and plotted against the test voltage. Raw currents were converted to conductances (G)

and the average and peak conductances were determined. Two-tailed, T-tests for independent groups were used for treatment comparisons. The alpha level was set at .05.

2.7 Na^+ Current Isolation and Characterization. For assessment of deactivation and inactivation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-1' solution containing (in mM) 70 NaCl, 92.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH. TTX (500 nM) was added to isolate $Na_v1.8$ from other Na_v that were present (Jiang and Cooper, 2011). The pipette solution contained 140 CsF, 10 NaCl, 5 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH.

For the deactivation test protocol, cells were held at -70 mV. An 8 ms step command to 0 mV was used to evoke a maximal Na^+ current. The cell was repolarized progressively in 10 mV steps ranging from -40 to -70 mV. As 8 ms was insufficient for full inactivation, a tail current could be measured during the repolarization step.

A time constant, tau_{deact}, was obtained by a successful fit to individual muscle and vascular nociceptors tail currents at each deactivation voltage. The mean and standard deviation of the tau_{deact} were determined by pooling across voltage for each cell type from vehicle and treatment conditions. Two-tailed, T-tests for independent groups were used for treatment comparisons with an alpha level set at .05.

The Na-Iso-1 solution containing 500 nM TTX was used to study the voltage dependence of steady state inactivation of $Na_v1.8$. Tissue specific nociceptive neurons were held at -70 mV. Starting at an inactivation conditioning step voltage of +10 mV, progressive command steps were made to -70 mV in 5 mV steps (400 ms). Each of these commands steps were followed by a fully depolarizing step to 0 mV (150 ms). The peak current evoked at 0 mV was measured and normalized against the maximum evoked current for that cell during the protocol (I/I_{MAX}). The normalized current was plotted against the conditioning step voltage and fit to a Boltzman type curve of the form:

$$I=I_{max}/(1+\exp((V_{.50}-V_m)/K))$$

where $V_{.50}$ is the voltage at which I is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{.50}$ was determined and then pooled for the various conditions. A two tailed T-test for independent groups was used to compare the $V_{.50}$ values. The 'p' value was set at .05.

3.1 Results

3.2 Influences of Chronic Neurotoxicants/PB on Pain Behaviors

Young adult male rats were treated with permethrin and chlorpyrifos along with a standard military dose of pyridostigmine bromide (assuming 70 kg body weight). After at least a 2 week acclimation period, treatments were initiated and continued for 60 days. Pressure pain measures were taken using a computer regulated, hand held test device by which pressure was applied via an 5 mm diameter ball transducer to the semitendinosus and biceps femoris muscles (right hind limb). To complement pressure pain testing, activity levels (movement distance, rest time) were recorded automatically in an activity box (15 min test period). Behavior tests were assessed in both treated (permethrin, chlorpyrifos, PB) and vehicle (ETOH, corn oil, water) exposed animals over an identical time course. Manual behavior testing occurred in double blinded conditions.

Although about 20% of soldiers deployed to the Persian Gulf developed GWI-like pain during their deployment, most developed symptoms following their return from the Gulf region (Kroenke et al., 1998). Accordingly behavior testing was focused on the post-exposure period. After a 60 day exposure to neurotoxicants, no reliable decrease in muscle pain threshold was observed. In contrast, consistent with lingering pain or illness, the amount of time spent resting during the 15 minute observation period was significantly increased at the end of neurotoxicant treatment (361.8 ± 17 vs 452.7± 16 sec; n=22). However, 8 and 12 weeks after treatments ceased, there were no significant differences in any of the pain or activity measures (Figure 1). Therefore, if any measurable pain resulted from the treatment with permethrin, chlorpyrifos and pyridostigmine bromide, it had resolved within 8 weeks.

Place figure 1 about here

3.3 Influences of Neurotoxicants and PB on Nociceptor Channel Protein Function

GWI pain is often described by veterans as a widespread pain in muscle, joints and other deep tissues (Stimpson et al., 2006; Thomas et al., 2006). As noted above, most war fighters reported chronic pain after their deployment had ended. Accordingly, we focused our molecular studies on nociceptive neurons that innervated deep tissues with extensive bodily representation (muscles and vessels), and on cellular and molecular changes that persisted long after exposure to neurotoxicants. Electrophysiological methods were used to examine the status of membrane proteins expressed in categories of muscle and vascular nociceptors harvested from treated and vehicle exposed animals 12 and/or 8 weeks after dosing had ceased (Rau et al., 2007; Rau et al., 2012).

3.4 Properties of $Na_v1.8$ Following Neurotoxicants/PB. TTXr $Na_v1.8$ is powerfully modified by acute permethrin administration (see Introduction; Jiang et al., 2013). In this report, the permethrin sensitive properties of $Na_v1.8$ were largely unaffected by a 60 day exposure to permethrin and the other agents. While acute permethrin produces >10 mV hyperpolarizing shift in $V_{1/2}$, no lasting influence on voltage dependence was observed after chronic exposure to NTPB (figure 2A-D); and in contrast to well established acute affects on the kinetics of deactivation (~2 fold increase; Jiang et al., 2013), there was no indication that the tau_{deact} of $Na_v1.8$ was modified in either mean duration or voltage dependence 8 weeks after a 60 day exposure to permethrin and other agents (figure 2E and F).

In contrast, $Na_v1.8$ decay constants (tau_{decay}), which are typically lengthened by pyrethroids (Jiang et al., 2013), remained elongated 8 weeks after administration of the neurotoxicant cocktail (figure 2A and C inserts). We assessed these decay constants from both activation (-20 mV test) and steady state inactivation protocols (10 mV test). In both of these independent analyses (separate cells), the tau_{decay} values were significantly lengthened for muscle nociceptor $Na_v1.8$. No changes were observed in vascular nociceptors following NTPB exposure. Despite the slowing of decay, the normalized peak $Na_v1.8$ current was not affected in muscle or in vascular nociceptors (I_{max}/C_m; data not shown). Nevertheless, the shift in decay constants could be expected to increase cellular excitability.

Some aspects of Na_v physiology (e.g., the voltage dependence of steady state inactivation) are not influenced by acute pyrethroid exposure (Tan and Soderlund, 2009; 2010; Jiang et a., 2013). Eight weeks after chronic exposure to NTPB, we did not observe changes in the voltage dependence of steady state inactivation in muscle or vascular nociceptors (figure 2). We did not examine $Na_v1.8$ physiology 12 weeks after treatment.

Place figure 2 about here

3.5 The Physiology of K_v7 Channel Proteins were Modified by Chronic NTPB. K_v7 proteins play an important role in the development of spontaneous activity. Originally referred to as M-current, these voltage sensitive K^+ channel proteins are often coupled to muscarinic acetylcholine receptors (Marion, 1997; Brown et al, 2005). Type 5 and type 8 muscle and vascular nociceptors express $K_v7.3$ (Jiang et al., 2013) and are sensitive to muscarine and/or the muscarinic agonist oxytremorine (Rau et al., 2012). The presence of two anticholinesterases (chlorpyrifos and PB) in the NTPB treatment regimen could affect the physiology of proteins that are coupled to muscarinic receptors. Accordingly we examined whether the activity of K_v7 proteins were influenced by a 60 day exposure to NTPB. Cells were harvested from treated and vehicle exposed rats 8 and 12 weeks following the treatments.

Potassium currents were isolated as described in 'Methods'. Using a classical 'current relaxation' procedure, the linopirdine sensitive K_v7 tail current (Jiang et al., 2013), was assessed across a series of deactivating voltage steps (see inset in figure 4b). The peak and average conductance was subsequently determined from the linopirdine sensitive current of neurons harvested 8 and 12 weeks after a 60 day exposure to permethrin and the anticholinesterases chlorpyrifos and PB.

Eight weeks after the treatment had ceased, we recorded significant increases in the normalized current and both the peak and average conductance of K_v7. These increases were only observed in vascular nociceptors (figures 3C and 4C). Significant increases in the normalized current (NTPB: 1.79 +/- .19 vs Vehicle: 1.15 +/- .17 pA/pF) and whole cell membrane resistance were also observed in vascular nociceptors at the 8 week delay point (NTPB: 423.5 +/- 46.5; Vehicle: 321.0 +/- 33.8 MOhms; $p<.05$, n=14 and 11 respectively; not shown). While changes in K_v7

physiology were intriguing, they did not persist over time. There were no significant shifts in K_v7 physiology at the 12 week delay period for either nociceptor subtype (figures 3 and 4).

We used current clamp methods to determine if the molecular influences of NTPB had functional consequences at the 8 week delay. Following a 6 minute exposure to linopirdine, action potentials were emitted spontaneously from both muscle and vascular nociceptors. Contrasting with findings for vehicle treated animals (above), the number of APs emitted by NTPB treated rats, were considerably higher (31.7 +/- 11.5 and 73.0 +/- 26.6 APs/min; n=8 and 8, muscle and vascular, respectively; figure 5B). The increase in spontaneous activity in type 8 vascular nociceptors was statistically significant relative to vehicle treated animals ($p<.04$; 11.25 +/- 6.8 and 14.8 +/- 5.5 APs/min; n= 4 and 8). Increases in spontaneous activity of muscle nociceptors failed to achieve significance ($p<.24$). These data suggest that, in the NTPB treated rats, K_v7 was suppressing a higher tendency for spontaneous AP discharge in vascular nociceptors. The factors potentiating discharge tendencies was not clear from this data. We repeated these tests at the 12 week observation interval when vascular nociceptor K_v7 currents had returned to normal levels. The six minute linopirdine treatment did not expose any heightened tendencies for spontaneous discharge at the 12 week delay.

Place figure 3 and 4 about here

In separate studies, we also examined whether the NTPB treated neurons exhibited any spontaneous activity in the absence of linopirdine (90 sec observation period). Spontaneous activity was observed in 3 treated vascular nociceptors at 22 C (41.3 +/- 25.8 APs; 3 of 11 cases). Vascular nociceptors in vehicle treated rats did not exhibit any spontaneous activity (n=10). When the bath temperature was raised to 35° C, 6 NTPB treated vascular nociceptors exhibited spontaneous activity while only 2 vehicle treated vascular nociceptors discharged (59.2 +/- 28.1 vs 4.0 +/- 3.5 APs; n= 9 and 10 respectively; $p<.05$). However 2 of the 6 vascular NTPB treated nociceptors discharged weakly (2 and 4 APs total). Muscle nociceptors were less reactive. Two of 6 muscle nociceptors discharged at 35° C (33 and 1 AP). None of the control

muscle nociceptors discharged at 35° C (n=3). There was no activity at 22° C in muscle nociceptors.

4.1 Discussion

Chronic, widespread joint and muscle pain is a frequent component of GWI syndromes (Stimpson et al., 2006; Thomas et al., 2006). It has been reported that the use of the nerve gas prophylactic, pyridostigmine bromide (PB) and a variety of insecticides/repellants co-varied with the development of Gulf War Illness (Binns et al., 2008; Steele et al., 2011). We examined whether chronic exposure to combinations of two neurotoxicants (chlorpyrifos and permethrin) with PB (NTPB) could produce a delayed chronic pain condition in rats; and whether corresponding cellular and molecular changes would occur in neurons coding for pain in muscle or vasculature. While some changes in the physiology of $Na_v1.8$ and K_v7 were observed and some were accompanied by functional changes in cellular activity, the molecular changes did not persist past the 8 week delay period, and no behavioral correlate of pain could be reliably measured from rats treated with NTPB.

Behavioral Studies. We attempted to develop a behavioral model of GWI pain using behavioral measures that could reflect chronic muscle (withdrawal threshold from applied pressure) or joint pain (reduced ambulation/increased rest). Other attempts to develop behavioral correlates of GWI, albeit with different treatment regimens, have focused on contiguous effects between neurotoxicants administered and on measures not clearly associated with pain (Abou-Donia et al., 2001; Abdel-Rahman et al., 2004; Abou-Donia et al 2004). During a 60 day exposure to NTPB, we observed increased resting behaviors that may have been associated with pain or malaise, but this only occurred during periods that coincided with neurotoxicant dosing. At the 8 week and 12 week delay periods, there was no indication of a change in hindlimb withdrawal or ambulatory behaviors of the treated rats. Accordingly, there was little indication that this behavioral model and exposure regimen could detect or quantify a widespread delayed onset pain of the sort reported by returning veterans (Kroenke et al., 1998; Stimpson et al., 2006; Thomas et al., 2006). While this did not rule out that some persistent pain, unmeasured with these methods, might nonetheless be present, the failure of the behavior model to measure any

pain in the weeks following NTPB exposure prevents any strong association of the molecular changes with a functional behavioral influence of these agents. Other behavioral measures and/or other treatment regimens may be required.

Molecular and Cellular Influences of NTPB Treatment. Acute exposure of permethrin to nociceptive neurons of the DRG, enhances voltage dependent activation, prolongs the decay kinetics, retards deactivation of $Na_v1.8$ and promotes spontaneous activity (Jiang et al., 2013). Most of these effects have been reported with other pyrethroids in various preparations (Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006). Because of its capacity to perturb an important nociceptor Na_v, permethrin exposure could play a key role in the development of the chronic pain of GWI. We hypothesized that the influence of chronic NTPB on $Na_v1.8$ would largely be reflective of the influence of permethrin. Other agents, that we co-administered, may have merely prolonged the effect or increased the absorption of permethrin (Baynes et al., 2002; Choi et al., 2004; Rose et al., 2005; see also Abu-Qare and Abou-Donia, 2008). After exposure of rats to NTPB for 60 days, evidence for specific permethrin-like molecular influences on $Na_v1.8$ were few (increased tau_{decay}) and differed between nociceptor subpopulations.

When examined 8 weeks after treatments, decay kinetics of type 5 muscle nociceptors reflected an influence of the 60 day exposure regimen. The increase in the tau_{decay}, was relatively modest (~1.3:1) as compared to what we observed with acute effects of permethrin (~3:1; Jiang et al., 2013); still, the fact that persistent molecular influences could be observed 8 weeks after exposure was potentially important. An increase in the tau_{decay} of $Na_v1.8$ could be expected to prolong action potential duration and possibly increase nociceptor excitability, but would not likely suffice as an initiator of AP discharge.

The main effect of extended exposure to NTPB was an increase in the conductance of K_v7 channel proteins. This occurred selectively in the vascular nociceptor group at the 8 week delay. When acutely applied, permethrin has no detectable influence on K_v7 physiology (Jiang, et al., 2013). Accordingly, it is difficult to tie the alterations in K_v7 reactivity directly to extended exposure to pyrethroids. K_v7 channels were historically identified as the source of 'M-currents' due to their regulation by muscarinic ACh receptors (Brown et al., 1997; Marion 1997). Disturbances in cholinergic physiology would not be surprising as Persian Gulf soldiers were

exposed to multiple anticholinesterases during their deployment. Exposures derived, not only as self-administered insecticides (i.e., chlorpyrifos) and nerve gas prophylactics (PB) but also from the intentional and/or accidental release of nerve agents (Sarin) and other carbamates or organophosphates with which they had voluntary and/or involuntary contact (Fricker et al., 2000; Haley et al., 2012; see Binns et al., 2008). The exposure to varied cholinesterase inhibitors might alter the physiology of cholinergic receptors and their downstream targets (K_v7); including those cholinergic receptors expressed on vascular nociceptors. The up-regulation of K_v7 current in vascular nociceptors could have been an adaptation to disturbed cholinergic regulation due to extended exposure to cholinesterase inhibitors. In recent reports, there is evidence of cholinergic vascular reflex anomalies in GWI veterans (Haley et al., 2009; Liu et al., 2010; Li et al., 2011). These reflex disturbances could be due to dysfunctional vascular afferents.

The presence of widespread, tonic pain in the Persian Gulf veterans suggested that K_v7 might deserve special scrutiny. Many of the types of pain reported by veterans would be considered tonic rather than evoked pain (Escalante and Fischbach, 1998; Proctor et al., 1998; Fukuda et al., 1998; Gray et al., 1999; Kang et al., 2000; Steele et al., 2000; Gray et al., 2002; Voelker et al 2002; Ang et al., 2006). K_v7 channel proteins play an important role in neuronal spontaneous discharge (Marrion, 1997; Brown and Passmore, 2009). The fact that large amounts of spontaneous activity were released by the K_v7 antagonist, linopirdine, suggests that other depolarizing currents were being restrained by the activity of K_v7 and that this restraint was more important after animals that were chronically exposed to NTPB. The enhanced magnitude of K_v7 conductance could represent a neuronal adaptation to suppress abnormal spontaneous discharge.

A less recognized influence of cholinesterase inhibitors, including PB and chlorpyrifos, is the increased expression of M_2 muscarinic receptors Abou-Donia et al., 2002;; Abou-Donia et al., 2003; Abou-Donia et al., 2004; but see Abu-Qare and Abou-Donia, 2003; Abdel-Rahman, et al 2004). There is also evidence of direct binding of the chlorpyrifos metabolite, chlorpyrifos-oxon, to muscarinic receptors (M2, M4; Huff and Abou-Donia, 1994; Huff and Corcoran, 1994; Howard and Pope, 2002). These muscarinic receptors are present and functionally active in rat DRG (Haberberger et al., 1999; 2000) and we have specifically observed that both type 5 and type 8 muscle and vascular nociceptors can be activated by muscarinic agonists (Rau et al.,

2012). It is not clear, by any known mechanism, how K_v7 expression might be tied to misregulation of muscarinic receptors.

Despite the interesting cellular and molecular changes we observed, these changes did not persist from the 8 week to the 12 week delay period, and no behavioral correlate of pain was recorded at either delay. If the molecular and functional derangements are limited to vascular nociceptors, it may have limited our capacity to measure behavioral correlates of this pain in the behavior test phase of the study. While it is possible that some pain was present, but could not be quantified by the test we used, the lack of molecular changes at the 12 week delay indicated that dosing conditions were not appropriate to create circumstances fully conducive for chronic pain. Nevertheless, the changes we observed could indicate how these agents, perhaps in the presence of other agents or circumstances, might achieve a persistence that would be sufficient to maintain molecular maladaptations.

If the enhanced conduction of K_v7 is suppressing a heighted tendency toward spontaneous discharge, then the source of that depolarizing flux remains to be determined. We have extensively examined $Na_v1.8$ without identifying changes consistent with increased depolarization. Both type 5 and type 8 nociceptors express TTXs channel proteins (possibly $Na_v1.7$) and the TTXr $Na_v1.9$ (Jiang and Cooper, 2011). TTXs channels are known targets of permethrin and could respond differently than TTXr channels to chronic exposure to NTPB. The actions of permethrin on $Na_v1.9$ are not known.

In conclusion, we have demonstrated that a mixture of permethrin, chlorpyrifos and PB can produce molecular adaptations in vascular and muscle nociceptors that last up to 8 weeks after exposure has ended. These changes include an increased tendency to emit spontaneous discharge, both at physiological temperatures and after 'release' of reactive inhibition of K_v7 channel proteins. Although the molecular defects subside by 12 weeks, the fact that we can observe persistent changes long after NTPB exposure is suggestive of a path to chronic pain.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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The funding agency had no role in the collection, analysis, or interpretation of the data. They did not participate in the writing of the report nor in the decision to submit the paper for publication.

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Figure Captions

Figure 1. Pain and Activity Measures were Unchanged 8 and 12 Weeks after NTPB Exposure lasting 60 Days. There were no behaviors effects in the post treatment period.

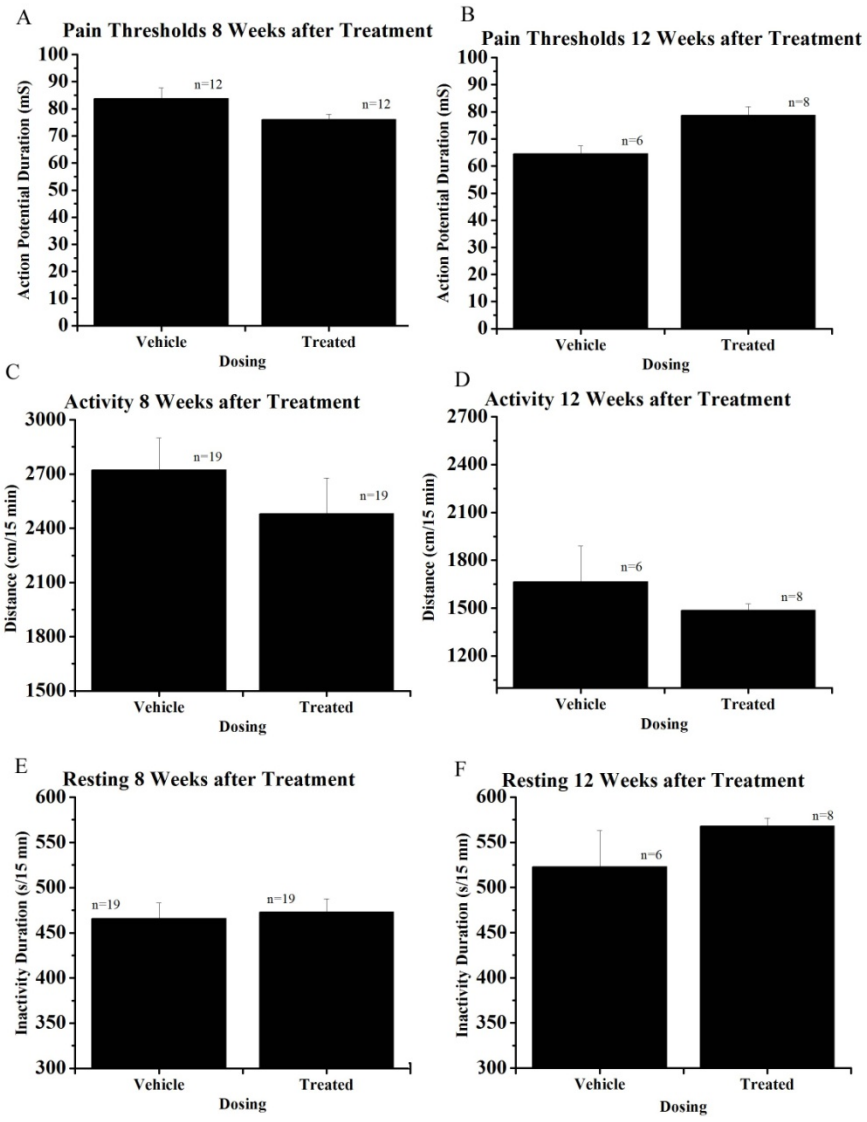
Figure 2. Voltage Dependent Activatio, Inactivation and Deactivation are Unchanged by NTPB Treatment. A and B) Muscle nociceptor activation and inactivation $V_{1/2}$ remained stable 8 weeks after a 60 day exposure to NTPB. Decay kinetics were signficantly longer in both tests (see insert taudecay; $p<.02$ and $p<.04$, A and B respectively). Representative traces are presented. **C and D)** Vascular nociceptor activation and inactivation $V_{1/2}$ remained stable 8 weeks after a 60 day exposure to NTPB. Decay kinetics were not altered (insert taudecay). Representative traces are presented (vehicle upper; NTPB lower). **E)** Vascular Nociceptors; **F)** Muscle nociceptors. Inserts present representative deactivation traces that are greatly shortened for visualization. Arrows indicate deactivation step.

Figure 3. Peak and Average Conductance of K_v7 are Increased 8 Weeks after NTPB. A and B) K_v7 currents are unchanged in muscle nociceptors at the 8 and 12 week delay. **C and D)** K_v7 current are increased in the 8 week delay but return to normal at the 12 week delay in vascular nociceptors ($p<.05$ and $p<.05$ for peak and average respectively).

Figure 4. Voltage Dependence of K_v7 Current 8 and 12 Weeks Following 60 Days of NTPB. **A and B)** The voltage dependence of the normalized current was unchanged in muscle nociceptors. **C and D)** The voltage dependence of the normalized current was unchanged in vascular nociceptors. The normalized current was significantly increased at -60 mV ($p<.03$, C). The increase of the normalized current did not persist for 12 weeks

Figure 5. Spontaneous Activity increased in Vascular Nociceptors from NTPB Treated Rats. A) When the bath temperature was increased to 35 C, more action potentials were admitted in vascular nociceptors from animals with NTPB exposure. **B)** Following application of linopirdine, significantly more APs are released from NTPB treated rats.

Figures and Tables



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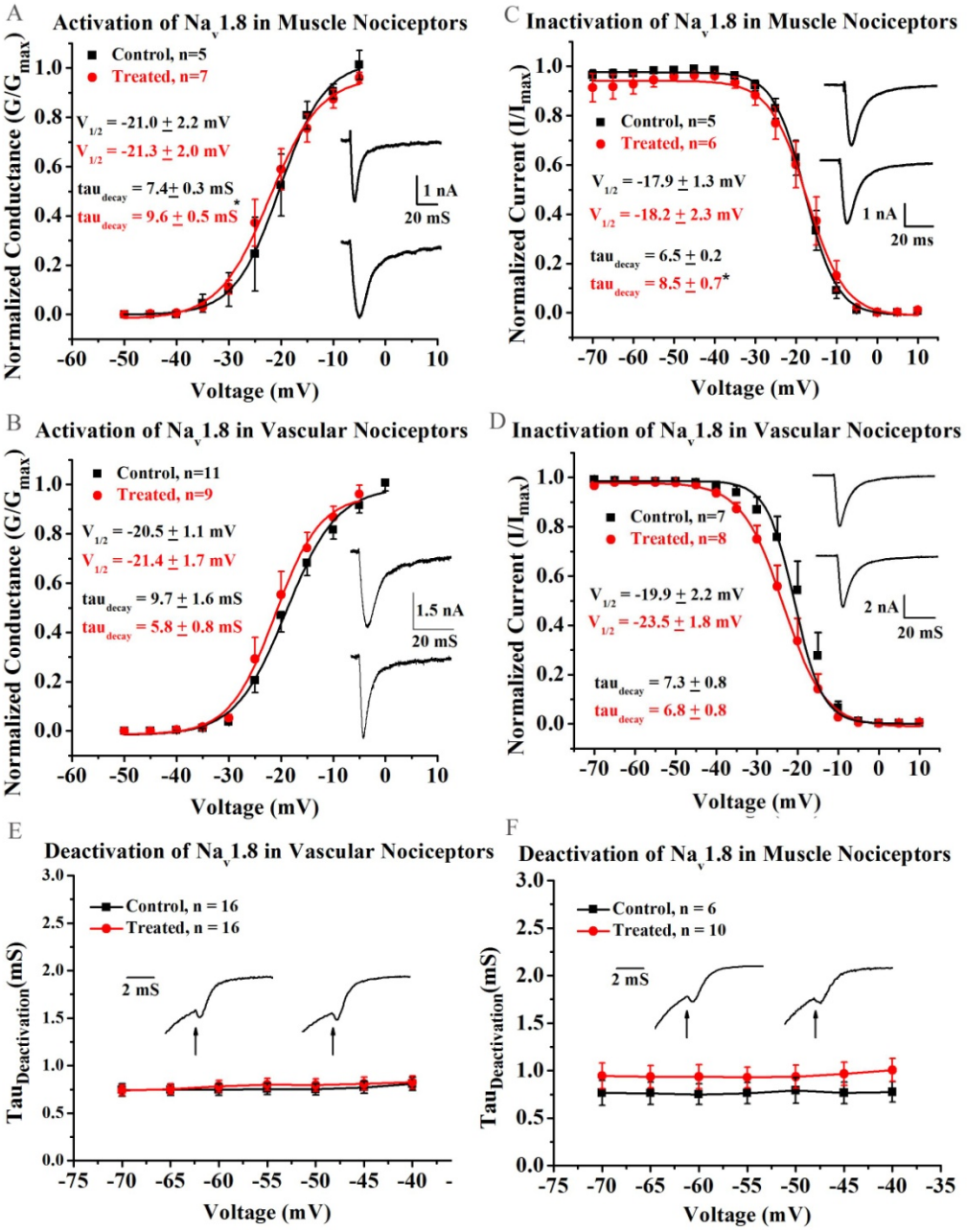

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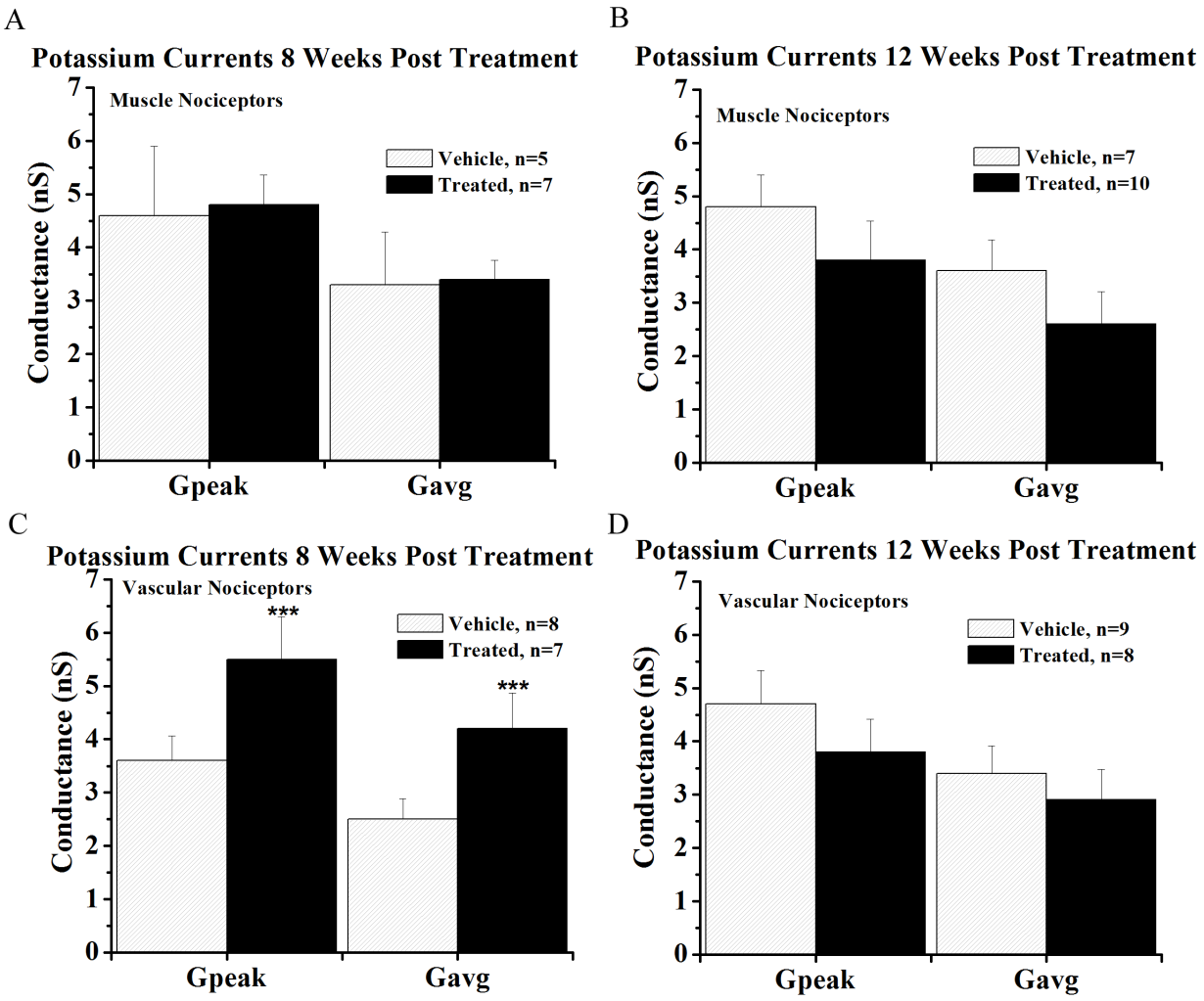

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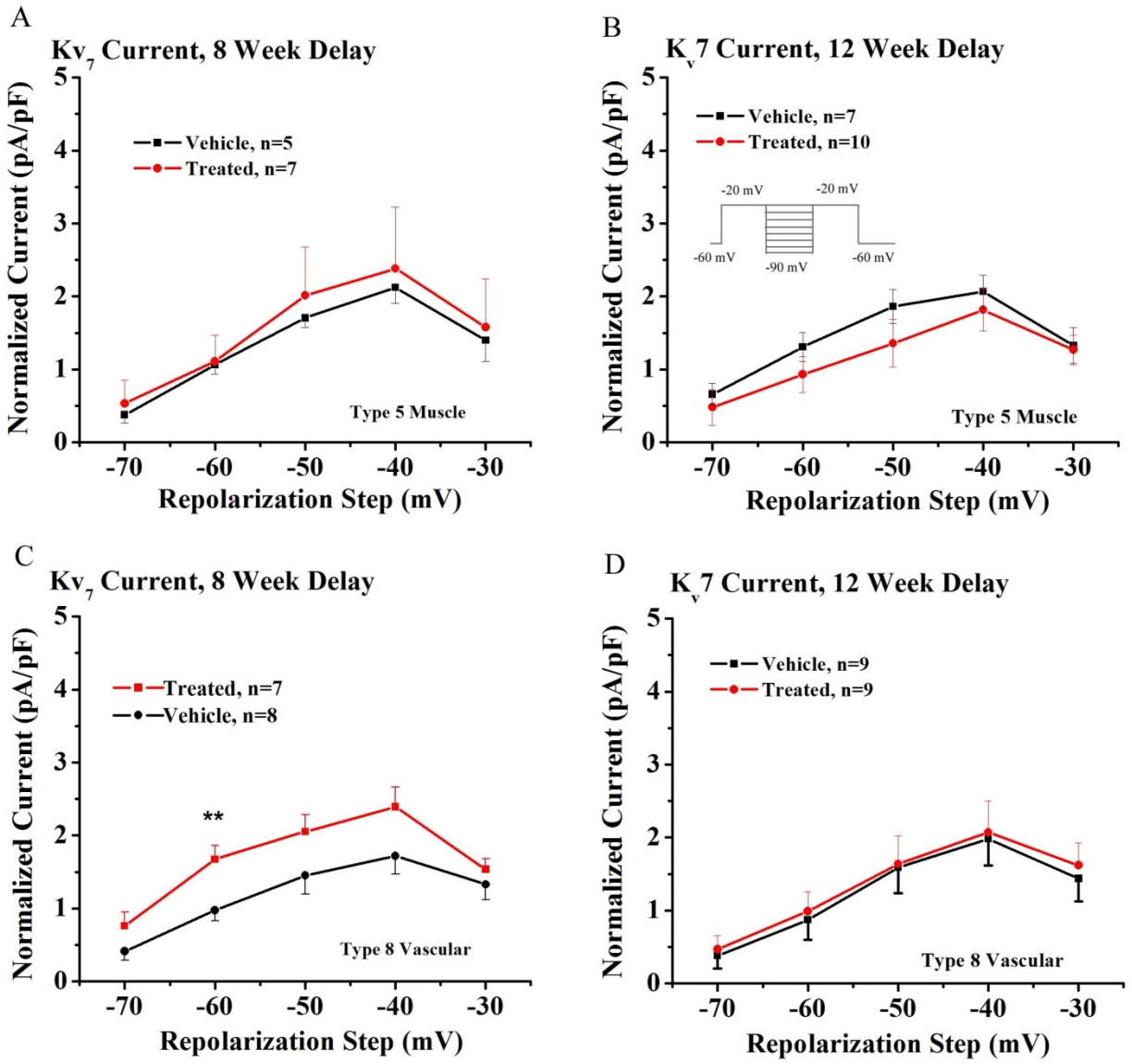

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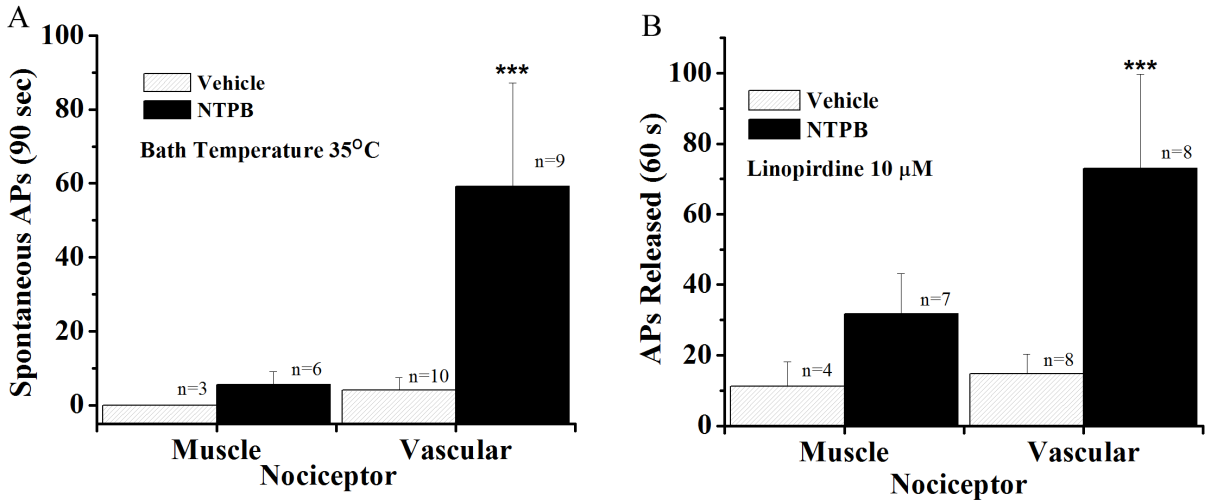

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